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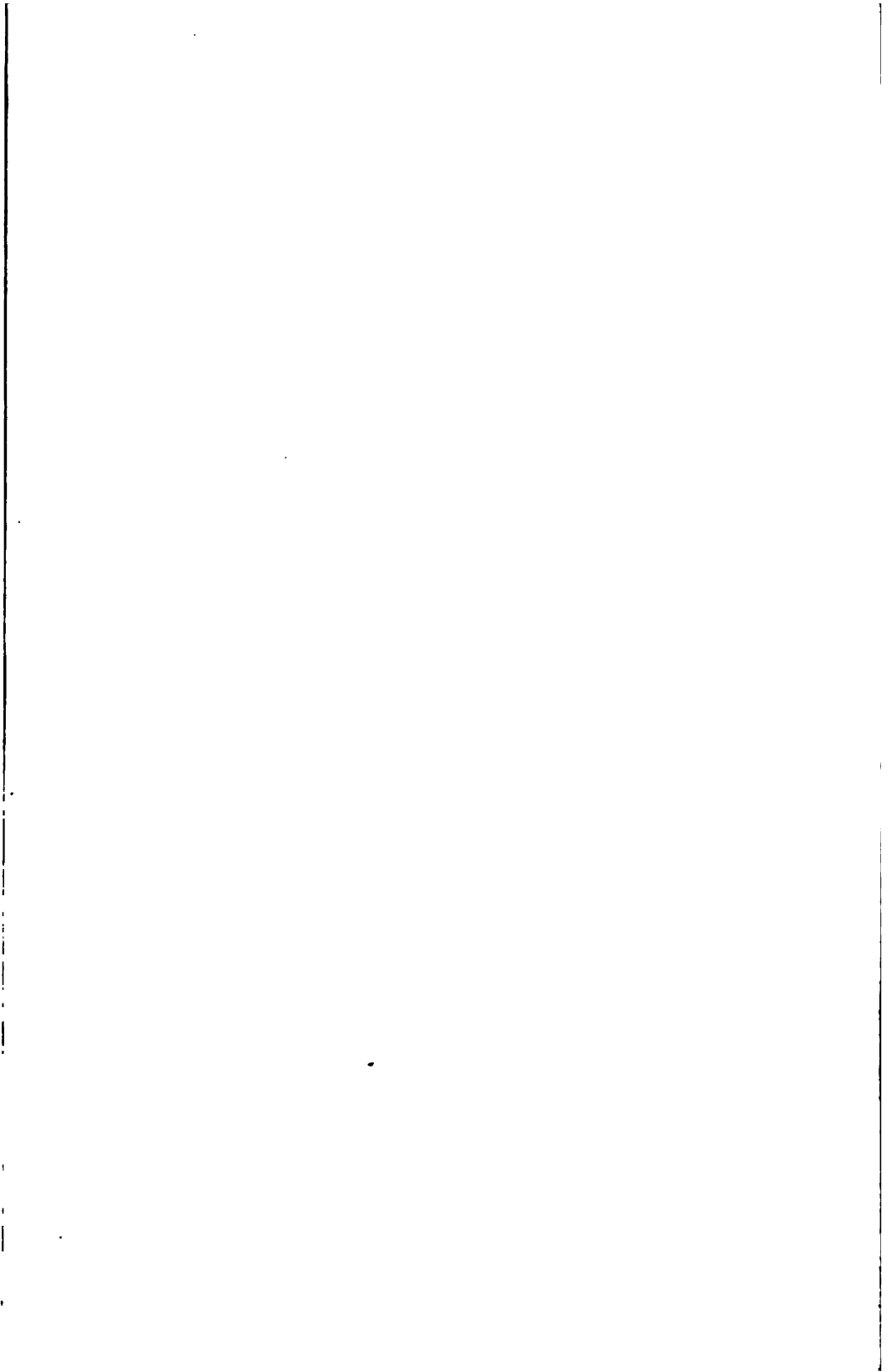
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CONTENTS TO VOLUME XXXVIII.

No. 1, MAY, 1919.

SUZUKI, NOBUYOSHI. Metabolism of the furan and hydrofuran derivatives in the animal organism.....	1
ANDO, HIDEZO. Behavior of cinnamic acid and its derivatives in the animal body.....	7
TSUDJI, MIDORI. Biological observations on the formation of phenol.....	13
HÖST, H. F. A study of the physiology of endogenous uric acid.....	17
OKEY, RUTH. Studies on the behavior of inulin in the animal body. Preliminary paper. Application of the Benedict method to the estimation of levulose and inulin.....	33
BLUNT, KATHARINE, and MALLON, MARGUERITE G. Digestibility of bacon.....	43
HAAS, A. R. C. Colorimetric determination of the hydrogen ion concentration in small quantities of solution.....	49
SUMNER, JAMES B. A rapid method for the estimation of urea in urine..	57
JOHNS, CARL O., and FINKS, A. J. Lysine as a hydrolytic product of hordein.....	63
HENDERSON, YANDELL, and STEHLE, RAYMOND L. Gas tensions in the tissues of the mouth.....	67
HAGGARD, HOWARD W., and HENDERSON, YANDELL. Gas tensions of the abdominal cavity, with some evidence on the diffusion of gases within the body.....	71
FOLIN, OTTO, and WU, HSIEN. A system of blood analysis.....	81
FOLIN, OTTO, and YOUNGBERG, GUY E. Note on the determination of urea in urine by direct Nesslerization.....	111
MCCOLLUM, E. V., SIMMONDS, N., and PARSONS, H. T. Biological analysis of pellagra-producing diets. VI. Observations on the faults of certain diets comparable to those employed by man in pellagrous districts. Plate I.....	113
EMMETT, A. D., and LUROS, G. O. Is lactalbumin a complete protein for growth?.....	147
TOYAMA, IKUZO. Relative abundance of serum proteins in albino rats at different ages.....	161
VAN SLYKE, DONALD D., STILLMAN, EDGAR, and CULLEN, GLENN E. Studies of acidosis. XIII. A method for titrating the bicarbonate content of the plasma.....	167
PATTEN, HARRISON E., and JOHNSON, ALFRED J. The effect of hydrogen ion concentration on the liquefaction of gelatine.....	179

No. 2, JUNE, 1919.

BIRCKNER, VICTOR. The zinc content of some food products.....	191
HILTNER, R. S., and WICHMANN, H. J. Zinc in oysters.....	205
OSBORNE, THOMAS B., and MENDEL, LAFAYETTE B. The nutritive value of yeast protein.....	223
FALK, K. GEORGE, MCGUIRE, GRACE, and BLOUNT, EUGENIA. Studies on enzyme action. XVII. The oxidase, peroxidase, catalase, and amylase of fresh and dehydrated vegetables.....	229
BIRCKNER, VICTOR. Acidimetric titration of grain extracts and amino-acids in the presence of alcohol.....	245
CLARK, E. P. Preparation of rhamnose.....	255
EMMETT, A. D., and LUROS, G. O. The stability of lactalbumin toward heat.....	257
HART, E. B., and STEENBOCK, H. Maintenance and production value of some protein mixtures.....	267
FOLIN, OTTO, and PECK, EUGENE C. A revision of the copper phosphate method for the titration of sugar.....	287
HESS, ALFRED F., and UNGER, LESTER J. The scurvy of guinea pigs. III. The effect of age, heat, and reaction on antiscorbutic foods..	293
HART, E. B., STEENBOCK, H., and SMITH, D. W. Studies of experimental scurvy. Effect of heat on the antiscorbutic properties of some milk products.....	305
EMMETT, A. D., and ALLEN, FLOYD P. Nutritional studies on the growth of frog larvæ (<i>Rana pipiens</i>). First paper.....	325
SAMPSON, JOHN J. The age at which trypsinogen appears in the fetal pancreas.....	345
FOSTER, M. G., and HOOPER, C. W. The metabolism of bile acids. I. A quantitative method for analysis of bile acids in dog's bile.....	355
FOSTER, M. G., HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. II. Normal fluctuations in healthy bile fistula dogs.....	367
FOSTER, M. G., HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. III. Administration by stomach of bile, bile acids, taurine, and cholic acid to show the influence upon bile acid elimination.....	379
FOSTER, M. G., HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. IV. Endogenous and exogenous factors.....	393
FOSTER, M. G., HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. V. Control of bile ingestion and food factors....	413
FOSTER, M. G., HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. VI. Origin of taurocholic acid.....	421

Contents

v

No. 3, JULY, 1919.

MORGULIS, SERGIUS, and JAHR, H. M. Determination of ammonia in the blood.....	435
GREENWALD, ISIDOR. A note on the determination of the inorganic constituents of blood and other physiological material.....	439
EMMETT, A. D., and LUKOS, G. O. The absence of fat-soluble A vitamine in certain ductless glands.....	441
DENIS, W., and MINOT, A. S. A method for determination of minute amounts of lead in urine, feces, and tissues.....	449
DENIS, W., and MINOT, A. S. The non-protein nitrogenous constituents of cow's milk.....	453
FOLIN, OTTO, and WU, HSIEN. A revised colorimetric method for determination of uric acid in urine.....	459
FOLIN, OTTO, and WRIGHT, L. E. A simplified macro-Kjeldahl method for urine.....	461
WILLIAMS, ROGER J. The vitamine requirement of yeast. A simple biological test for vitamine. Plate 6.....	465
FENGER, FREDERIC, and HULL, MARY. Relationship of certain pancreatic enzymes.....	487
NORGAARD, A. V. S. Studies of the concentration of catalase in urine, chyme, and feces.....	501
HART, E. B., and HUMPHREY, G. C. Can "home grown rations" supply proteins of adequate quality and quantity for high milk production.....	515
KENDALL, E. C. The use of turpentine resin in turpentine as a foam breaker.....	529
MCCLENDON, J. F., and SHARP, PAUL F. The hydrogen ion concentration of foods.....	531
MCCLENDON, J. F., MYERS, FRANK J., CULLIGAN, LEO C., and GYDESEN, CARL S. Factors influencing the hydrogen ion concentration of the ileum.....	535
MCCLENDON, J. F., VON MEYSENBUG, L., ENGSTRAND, O. J., and KING, FRANCES. Effect of diet on the alkaline reserve of the blood....	539
MCCLENDON, J. F., and PRENDERGAST, H. J. Note on the ultramicroscopy of egg albumin.....	549
Index to Volume XXXVIII.....	551

METABOLISM OF THE FURAN AND HYDROFURAN DERIVATIVES IN THE ANIMAL ORGANISM.

By NOBUYOSHI SUZUKI.

(From the Laboratory of Medicine, Kyoto Imperial University, Kyoto.)

(Received for publication, February 15, 1918.)

Production of Hydroxymethylpyromucic Acid from Chitose.

Studies of the intermediary products of catabolism have made remarkable progress since Knoop¹ in 1904, employed aromatic fatty acids in his experiments. The aliphatic fatty acids, which are highly resistant to the action of most chemical reagents, are completely oxidized in the animal organism into their end-products, carbon dioxide and water. On the other hand, the benzene nucleus is strongly resistant to oxidation *in vivo*, and the fate of aromatic fatty acid can be followed in the animal organism. The furan nucleus is quite as refractory in the animal organisms as the benzene nucleus, and the furan compounds are in some respects closely related to natural substances, especially carbohydrates.

Jaffé and Cohn,² giving furfural to animals, found that this substance had been changed *in vivo* into furfuralacrylic acid in a manner quite analogous to Perkins' synthesis, while on the other hand, it also gave pyromucic acid by oxidation. These were coupled with glycocoll and excreted as furfuralacrylic acid and pyromucic acid. Furthermore, Sasaki³ observed that furfuralpropionic acid is changed in the animal body into furfuralacrylic acid and pyromucic acid. From this fact it has been ascertained that the saturated fatty acids change into α - β -unsaturated acids. Oxymethylfurfural⁴ is also easily produced from cellulose or from saccharides.

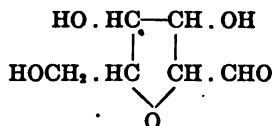
¹ Knoop, F., *Der Abbau aromatischer Fettsäuren im Tierkörper*. Monograph, Freiburg, 1904; *Beit. chem. Physiol. u. Path.*, 1905, vi, 150.

² Jaffé, M., and Cohn, R., *Ber. chem. Ges.*, 1887, xx, 2311.

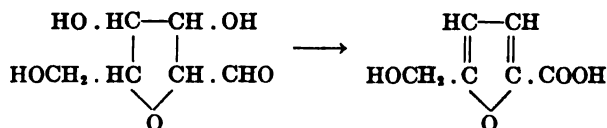
³ Sasaki, T., *Biochem. Z.*, 1910, xxv, 272.

⁴ Erdmann, E., *Ber. chem. Ges.*, 1910, xliii, 2391. Van Ekenstein, W. A., and Blanksma, J. J., *ibid.*, 2355.

Chitose is prepared from glucosamine hydrochloride by Fischer and Tiemann's⁵ method. Fischer and Andreae⁶ recognized it as a hydrofuran derivative with the following constitution:

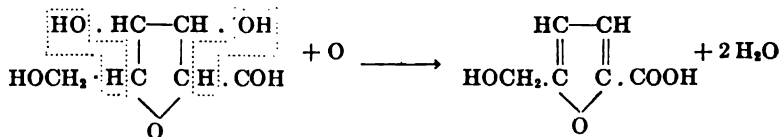


Thus glucosamine is often considered a derivative of chitose and called chitosamine. Though chitose is not properly to be regarded as a sugar, it resembles a monosaccharide in some respects. Under the direction of Professor Sasaki, I have studied the metabolism of chitose in the animal organism. I have been able to isolate from the urine of animals to which chitose had been given, a substance which crystallizes especially well, and I have identified it as hydroxymethylpyromucic acid.



Instances are not wanting of aldehyde groups being oxidized to carboxylic acids in the animal organism, but the production of a furan compound, that is, the double linkage at α - β -position, is worthy of note.

Many hydroxyl acids change into unsaturated acids corresponding to them, *e.g.*, malic acid into fumaric acid, citric acid into aconic acid, and mucic acid into furfurandicarboxyl acid (dehydromucic acid⁷). The conversion of chitose into hydroxymethylpyromucic acid can be represented as follows:

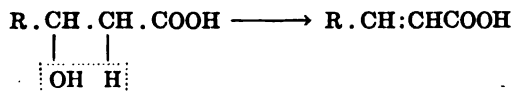


⁵ Fischer, E., and Tiemann, F., *Ber. chem. Ges.*, 1894, xxvii, 139.

⁶ Fischer, E., and Andreae, E., *Ber. chem. Ges.*, 1903, xxxvi, 2589.

⁷ Seelig, E., *Ber. chem. Ges.*, 1879, xii, 1083. Fischer, E., *ibid.*, 1891, xxiv, 2140. Hill, H. B., *ibid.*, 1899, xxxii, 1221. Heinzelmann, R., *Ann. Chem.*, 1878, cxci, 184.

This result is in accord with the view that α - β -unsaturated acids are derived from hydroxyl acids in the body.



Thus Dakin⁸ has isolated cinnamoylglucosyl by giving phenyl- β -oxypropionic acid to dogs.

The author is making further studies of chitonic acid, isosaccharic acid, and oxymethylfurfural, and expects to report them later.

EXPERIMENTAL.

Chitose cannot be crystallized, and since it cannot be prepared in pure form, the following experiment was first made. 15 gm. of chitose syrup were dissolved in water, acidified by addition of phosphoric acid, and extracted with ether using the Kumagawa-Suto liquid extractor for 6, 10, and 40 hours. Only a small quantity of syrup was obtained from each of the extracts, but no crystalline substance. Thus, the 15 gm. of chitose syrup used contained no crystalline furan compounds, and certainly no hydroxymethylpyromucic acid.

Chitose was given to rabbits which had been fed with "okara"⁹ by injecting its solution subcutaneously, or giving it by stomach tube. The urine of the animals was collected for 2 days after the chitose was given and evaporated to a thin syrup, which was extracted thoroughly with hot 90 per cent alcohol. The evaporated alcoholic extract was dissolved in water, acidified with phosphoric acid, and extracted with ether in the Kumagawa-Suto extracting apparatus. From the first (10 hours) and the second (15 hours) ether extracts, after the evaporation of ether, a beautifully crystalline substance was obtained. From the third (40 hours) and the fourth (20 hours) fractions, a syrup was obtained, but no crystalline substance could be isolated. The crystalline substances from the first and second extracts were purified on a

⁸ Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 213.

⁹ Refuse of beans produced in the manufacture of "tofu" which is made from beans strained through rough cloth, after they have been boiled soft and ground into a semiliquid substance.

TABLE I.

Experiment No.	Sex.	Weight of rabbit.	Quantity of chitose.	Method.	Total quantity of urine.	Substance obtained	Per cent of substance obtained to that given.
		kg.	gm.		cc.	gm.	per cent
I	♀	2.56	10	Dissolved in 15 cc. water + 2 gm. sodium carbonate. <i>Per os.</i>	1st day, 360 2nd " 290	0.782	7.82
II	♀	2.22	15	Dissolved in 25 cc. water. <i>Per os.</i>	1st day, 100 2nd " 90	0.895	5.97
III	♀	2.25	15	Dissolved in 25 cc. water + 2 gm. Na ₂ CO ₃ . <i>Per os.</i>	1st day, 160 2nd " 120	0.983	6.55
IV	♂	2.55	8 { Portion I, 3 Portion II, 5	I. Portion dissolved in 6 cc. water. II. Portion dissolved in 10 cc. water. Subcutaneously.	1st day, 250 2nd " 210 3rd " 180	0.572	7.15
V	♀	2.45	10 { Portion I, 5 Portion II, 5	Dissolved in 10 cc. water. 1 gm. sodium carbonate. Subcutaneously.	1st day, 200 2nd " 185 3rd " 220	0.832	8.3
VI	♀	3.00	40 in four portions; 10 daily.	Dissolved in 20 cc. water. 2 gm. sodium carbonate. <i>Per os.</i>	1st day, 285 2nd " 220 3rd " 160 4th " 200 5th " 150	1.72	4.3
VII	♂	3.80	20 in four portions; 5 daily.	Dissolved in 10 cc. water. 1 gm. sodium carbonate. Subcutaneously.	1st day, 260 2nd " 225 3rd " 230 4th " 150 5th " 215	1.08	5.04

clay plate. The crystals were then recrystallized from boiling water after adding animal charcoal. The beautiful, fine, colorless, needle-shaped crystals were then dried in a vacuum desiccator over sulfuric acid and weighed. The results of seven experiments with animals are shown in Table I.

The percentages of the quantity of the crystals obtained from the urine of the animals to which chitose had been given, to the amounts of chitose given were from 4.3 to 8.32. The substance melted at 165–167° (uncorrected), turning brown and evolving gas. It dissolves readily in water, alcohol, and glacial acetic acid, but is difficultly soluble in ether, and almost wholly insoluble in benzene, chloroform, and carbon disulfide. For analysis it was again dissolved in water, to free it entirely from animal charcoal, and after addition of phosphoric acid, again extracted with ether. The crystals obtained were then recrystallized from water and dried over sulfuric acid in a vacuum desiccator; they were further dried at 100°, until their weight became constant.

0.1811 gm. substance gave 0.3346 gm. CO_2 and 0.0685 gm. H_2O .

	Calculated for $\text{C}_8\text{H}_8\text{O}_4$	Found:
C.....	50.69	50.39
H.....	4.26	4.23

The properties of the acid were identical with those recorded by Hill and Jennings¹⁰ and by Fischer and Andreae.⁶

¹⁰ Hill, H. B., and Jennings, W. L., *Am. Chem. J.*, 1893, xv, 181.

BEHAVIOR OF CINNAMIC ACID AND ITS DERIVATIVES IN THE ANIMAL BODY.

By HIDEZO ANDO.

(From the Laboratory of Medicine, Kyoto Imperial University, Kyoto.)

(Received for publication, February 15, 1918.)

Magnus-Levy¹ has proved by experiment the fact that the α -amino-acids, which generally are readily burnt up in the animal organism, escape destruction if the amino group is benzoylated. At the suggestion of Professor Sasaki, I have tried to determine whether the unsaturated benzoylated amino-acids would remain unattacked in the animal body. Experiments were made on dogs and rabbits with benzoyl- α -aminocinnamic acid, with *p*-hydroxybenzoyl- α -aminocinnamic acid, and with cinnamoyl tyrosine. It was found that benzoyl- α -aminocinnamic acid was almost quantitatively eliminated in the urine unchanged. On the other hand, *p*-hydroxybenzoyl- α -aminocinnamic acid could be found in the urine after subcutaneous administration only, and in varying and reduced quantity. Only once an insignificant percentage was found in the urine after administration by mouth.

This difference as to the excretion of the two compounds of similar chemical constitution led us to try the same experiment with benzoyl- α -aminocinnamic acid. This also was excreted in the urine almost quantitatively after administration by mouth or subcutaneously. Cinnamoyl tyrosine, given to dogs, appeared to be completely oxidized, since no trace of it was found in the urine. In rabbits, however, after subcutaneous administration it could be traced in minimal quantities in the urine; when given by mouth, hippuric acid could be isolated from the urine, but no other derivative. Unlike benzoyl tyrosine, therefore, cinnamoyl tyrosine is further split up in the animal body.

¹ Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 555.

<i>Substance.</i>	<i>Formula.</i>	<i>Fate.</i>
Benzoyl- α -amino- <i>p</i> -hydroxycinnamic acid.	$C_6H_4(OH).CH : C(COOH).NH.CO.C_6H_5$	Chiefly decomposed.
Benzoyl- α -amino- cinnamic acid.	$C_6H_5.CH : C(COOH).NH.CO.C_6H_5$	Excreted unchanged.
Benzoyl- <i>o</i> -amino- cinnamic acid.	$C_6H_5.CO.NH.C_6H_4.CH : CH.COOH$	" "
Cinnamoyl tyrosine.	$OH.C_6H_4.CH_2.CH(COOH)NH.CO.CH : CH.C_6H_5$	Decomposed.

EXPERIMENTAL.

Benzoyl- α -aminocinnamic acid and *p*-hydroxybenzoylamino-cinnamic acid were prepared according to Erlenmeyer's² directions. Benzoyl-*o*-aminocinnamic acid was obtained synthetically by benzoylating *o*-aminocinnamic acid, and was found to contain the proper percentage of nitrogen.

The cinnamoyl-*l*-tyrosine has not been synthesized previously and was prepared as follows:

(a) 25 gm. of tyrosine ethyl ester hydrochloride were suspended in 250 cc. of chloroform, cooled to 0°C., and 103 cc. of normal sodium hydroxide were added. The tyrosine ester thus liberated went into the chloroform.

(b) 20 gm. of cinnamoyl chloride were dissolved in 125 cc. of anhydrous chloroform.

(c) Approximately 12 gm. of dry sodium carbonate were dissolved in 50 cc. of water. Half of the cinnamoyl chloride solution was added to the cooled tyrosine ester-chloroform solution mentioned above, and the mixture thoroughly shaken. Part of the tyrosine ester combines with liberated hydrochloric acid, forming the insoluble hydrochloride. A little of the sodium carbonate solution was added and the shaking continued. The re-

² Erlenmeyer, E., *Ann. Chem.*, 1893, cclxxv, 1.

mainder of the cinnamoyl chloride and the carbonate solutions was now alternately added in small amounts; the mixture was constantly shaken and kept cool. The chloroform solution was then dried with sodium sulfate and precipitated with much petroleum ether. The voluminous and cloudy precipitate is at first pasty, but changes gradually into crystalline sediment. This was dissolved again in chloroform, and after repeated precipitation with petroleum ether, finally recrystallized with dilute alcohol. Its melting point is 136°C. (uncorrected). For analysis the substance was then dried at 100°.

0.1700 gm. substance gave 4.7 cc. 0.1 N NH_3 .

	Calculated for $\text{C}_{10}\text{H}_{11}\text{O}_2\text{N}$:	Found:
N.....	4.13	3.87

The saponification of the ester was accomplished by simply dissolving the substance in N NaOH. By neutralizing with hydrochloric acid the cinnamoyl tyrosine was precipitated as an amorphous mass. The pure substance, polygonal prisms, obtained by crystallizing from dilute alcohol, melted at 166–167°C. (uncorrected), and was dextrorotatory in alkaline solution. The substance was dried at 100°.

0.2576 gm. substance gave 15.5216 gm., dissolved in about 15 cc. N NaOH; specific gravity 1.0447; percentage 1.6596; length of tube, 1 dm. $\alpha = +0.66$ at 25°C. Therefore $[\alpha]_D = +38.07^\circ$.

0.1973 gm. substance gave 0.5039 gm. CO_2 and 0.0980 gm. H_2O .

0.1256 " " " 3.8 cc. 0.1 N NH_3 .

	Calculated for $\text{C}_{18}\text{H}_{17}\text{O}_2\text{N}$:	Found:
C.....	69.42	69.65
H.....	5.51	5.53
N.....	4.50	4.24

The cinnamoyl-L-tyrosine is readily soluble in alcohol, slightly in ether, and scarcely soluble in water.

As experimental animals, dogs and rabbits were employed. The substances to be tested were used in the form of a weak alkaline carbonate solution and were administered subcutaneously or introduced into the stomach. Only in dogs were the substances given *per os* as powder wrapped in meat. The urine of 24 or 48 hours was concentrated over steam and extracted with alcohol.

Cinnamic Acid

TABLE I.
Benzoyl- α -Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	<i>kg.</i>		<i>gm.</i>	<i>gm.</i>	
Dog.....	14.6	Subcutaneous.	2.0	0.863	—
".....	8.2	"	2.0	1.215	—
".....	14.6	<i>Per os.</i>	2.0	1.300	—
Rabbit.....	2.1	Subcutaneous.	0.7	0.321	—
".....	2.3	"	1.5	0.722	—
".....	2.7	<i>Per os.</i>	1.0	0.687	—

TABLE II.
p-Hydroxybenzoyl- α -Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	<i>kg.</i>		<i>gm.</i>	<i>gm.</i>	
Dog.....	8.20	Subcutaneous.	2.0	0.554	—
".....	3.10	"	1.0	0.276	—
Rabbit.....	3.00	"	0.7	—	—
Dog.....	3.50	<i>Per os.</i>	2.0	0.013	—
Rabbit.....	2.95	" "	1.0	0.120	—
".....	3.00	" "	1.25	—	—
".....	2.61	" "	1.5	—	—

TABLE III.
Benzoyl-o-Aminocinnamic Acid.

Animal.	Weight.	Method.	Amount given.	Immediately after acidification.	Found in ether extract.	Optically active substances in mother liquor.
	<i>kg.</i>		<i>gm.</i>	<i>gm.</i>		
Dog.....	4.5	Subcutaneous.	1.0	0.503	Minimal.	—
".....	6.8	"	2.0	1.266	"	—
Rabbit.....	2.3	"	0.7	0.360	—	—
Dog.....	4.5	<i>Per os.</i>	1.5	0.497	Minimal.	—
Rabbit.....	2.4	" "	1.0	0.630	—	—

TABLE IV.
Cinnamoyl-L-Tyrosine.

Animal.	Weight.	Method.	Amount given.	Found in ether extract.	Optically active substances in mother liquor.
	<i>kg.</i>		<i>gm.</i>	<i>gm.</i>	
Dog.....	12.1	Subcutaneous.	2.0	—	—
"	7.9	<i>Per os.</i>	2.0	—	—
"	7.9	" "	2.0	—	—
"	6.7	" "	2.0	—	—
Rabbit.....	2.7	Subcutaneous.	1.0	0.07	—
"	3.3	"	0.7	—	—
"	2.6	<i>Per os.</i>	1.0	0.306	—
"	2.4	Subcutaneous.	0.8	0.245	—
"	2.7	<i>Per os.</i>	2.0	0.383	—
"	3.1	" "	2.0	0.572	—
"	3.3	" "	2.0	0.51	—
"	2.7	" "	2.0	—	—

The alcoholic extract was in turn evaporated and the residue dissolved in water. The solution, strongly acidified with hydrochloric acid, was first thoroughly extracted with ether in the Kumagawa-Suto extractor. The resulting acid residue was then shaken with ethyl acetate. From this extract, only urea was isolated. The mother liquors contained no optically active substances. The substances found were identified by their physical properties and especially by determining their melting point. The mixing test with analytically pure substance was performed each time.

The substance found in the ether extract was recrystallized from hot dilute alcohol and purified. The figures given thus indicate the quantity of analytically pure benzoyl- α -aminocinnamic acid.

BIOLOGICAL OBSERVATIONS ON THE FORMATION OF PHENOL.

By MIDORI TSUDJI.

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(Received for publication, February 15, 1918.)

The formation of phenols in the animal body is a well known fact. They are known as the putrefaction products of the pancreas, muscle, liver, and fibrin.¹ However, the phenols as judged by the reaction with bromine water are not a single substance, but include *p*-cresol, *o*-cresol, *m*-cresol, and phenol. Baumann and Brieger² have shown that the phenols produced on putrefaction of the liver are for the most part *p*-cresol, *o*-cresol, and a little phenol. It was obvious that the mother substance of phenol might be tyrosine, and though Baumann failed in one of his experiments, Brieger,³ who administered tyrosine to a healthy man, was successful in showing by the bromine water test much phenol in the urine. Finally, Weyl,⁴ on adding sewer mud to tyrosine, confirmed that its putrefaction product was one of the phenols, indeed *p*-cresol itself. Hence, Baumann,⁵ thinking that phenol should arise by oxidation of *p*-cresol, administered *p*-cresol to a dog and *p*-oxybenzoic acid was obtained in the urine; also, after feeding *p*-oxybenzoic acid to the same dog, he obtained phenols in the urine. After subjecting hydroparacumalic acid⁶ (*p*-oxyphenolpropionic acid) to putrefactive fermentation with pancreas substance, he obtained *p*-oxyphenylacetic acid, *p*-cresol, and phenol. Since hydroparacumalic acid is the decomposition

¹ Baumann, E., *Z. physiol. Chem.*, 1877, i, 60. Brieger, L., *ibid.*, 1879, iii, 134. Odermatt, W., *J. prakt. Chem.*, 1878, xviii, 249. Nencki, M., *Opera omnia*, Braunschweig, 1905, i, 404.

² Baumann, E., and Brieger, L., *Z. physiol. Chem.*, 1879, iii, 149.

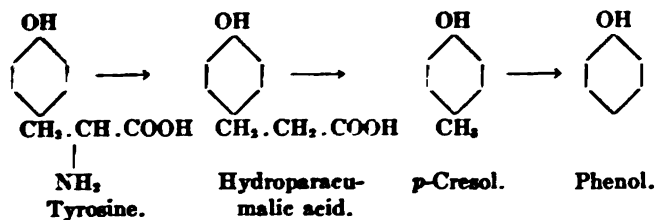
³ Brieger, L., *Z. physiol. Chem.*, 1878, ii, 241.

⁴ Weyl, T., *Z. physiol. Chem.*, 1879, iii, 312.

⁵ Baumann, E., *Z. physiol. Chem.*, 1879, iii, 250.

⁶ Baumann, E., *Z. physiol. Chem.*, 1880, iv, 304.

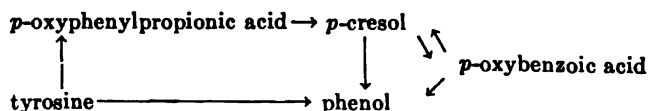
product of tyrosine, Baumann considered the formation of phenol as follows:



Nevertheless, Baumann could not directly derive phenol from *p*-cresol; also, in testing for phenol in the urine of a dog which had been given *p*-oxybenzoic acid, he used the reaction with bromine water. It was therefore not certain whether the decomposition product was phenol or not, as it could also be *p*-cresol. Fortunately an important contribution was furnished by Siegfried and Zimmermann,⁷ who, after giving *p*-oxybenzoic acid to dogs, obtained 32 per cent each of phenol and *p*-cresol in the urine.

Although the production of phenol from both *p*-cresol and *p*-oxybenzoic acid has thus been proved, we must not come too readily to the conclusion that phenol is obtained from hydroparacumalic acid, because by fermentation of certain organs containing none of it, phenol is produced.

Judging from the structural formula, the mother substance of phenol seems to be tyrosine, but we must note that phenylalanine is also capable of being the mother substance of phenol. When Baumann put forward his hypothesis, phenylalanine had not yet been identified as a protein constituent. On studying phenol formation by organisms, I found unexpectedly *Bacillus coli communis*, which actively forms phenol. I studied its action on tyrosine and phenylalanine; no phenol was formed from phenylalanine; however, much phenol but no cresol was obtained from tyrosine. From these experimental results, phenol formation may be represented thus:



⁷ Siegfried, M., and Zimmermann, R., *Biochem. Z.*, 1912, xlv, 210.

Baumann assumes hydroparacumalic acid to be concerned in phenol formation, but it must be admitted that there remains room for further investigation. We have among the decomposition products of tyrosine so called tyrosol, *p*-oxyphenyllactic acid, and tyramine; the possibility of these substances being concerned in phenol production must be considered.

EXPERIMENTAL.

Various organisms were examined as to phenol production after being grown for 30 days in bouillon containing 2 per cent peptone (Witte). The methods employed for testing for phenols were: Millon's reaction, bromine water, and ferric salts reaction. The tests were made on distillates derived from the cultivating fluid, which had been acidified by addition of 10 cc. of dilute sulfuric acid and distilled with steam.

TABLE I.
30 Days.

Bacillus.	No. of strain.	Phenols.
<i>B. proteus vulgaris</i>	1	—
" " ".....	2	—
" " ".....	3	—
" <i>typhosus</i>		—
" <i>paratyphosus</i>		—
" <i>coli communis</i>	1	+++
" " ".....	2	—
" <i>subtilis</i>		—
" <i>pyocyaneus</i>		—

The results in Table I show that *Bacillus coli* is the only organism among those tested that produced noteworthy amounts of phenol. In a second investigation, it was found that *Bacillus coli* forms significant amounts of phenol after 2 days, and that its quantity subsequently increases.

To determine whether phenylalanine could yield phenol, the following experiment was made. A nutrient medium containing glycerol and the usual inorganic salts was used with 0.5 gm. of phenylalanine or 1.0 gm. of tyrosine per liter. It was heavily inoculated with *Bacillus coli* from twenty agar slants and then

incubated. After 30 days, the culture was still pure and the mixtures were acidified and distilled. The phenylalanine experiment gave no trace of phenol, while tyrosine gave much. It may be inferred from these results that the phenols come from tyrosine, while *dl*-phenylalanine does not contribute to them. Subsequent experiments made under both aerobic and anaerobic conditions only confirmed the conclusion.

In order to identify the phenol formed from tyrosine, and present in the acid distillates, an attempt was made to convert it into the barium salt of phenylsulfuric acid. The amount obtained was too small for analysis, so that the isolation of phenol itself was next attempted. The distillate was made alkaline with potash, evaporated, acidified with sulfuric acid, and extracted with ether. The ether was evaporated at low temperatures, and on cooling the residue in an ice box, needles of phenol, melting at 42–43°, were readily obtained. The yield was only about 30 mg., but the product showed an unchanged melting point when mixed with pure phenol and gave all the characteristic reactions of the latter substance.

In conclusion, I wish to acknowledge my indebtedness to Professor Sasaki for his interest and advice during the progress of these studies.

A STUDY OF THE PHYSIOLOGY OF ENDOGENOUS URIC ACID.

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(Received for publication, October 28, 1918.)

Our knowledge of the physiology of endogenous uric acid is still incomplete. Aside from questions concerning the accumulation of uric acid in blood, about which opinions differ, little is known of the concentration of uric acid in blood. It is not certain whether the concentration of the endogenous uric acid in blood is a constant factor for the individual, or whether it varies, and if so, whether the variation during the day is regular or irregular.

The amount of endogenous uric acid in the urine has been the subject of many investigations, which have resulted in two different opinions. The majority of the authors emphasize the fact that the output of the endogenous uric acid in 24 hours is constant for the individual (Burian and Schur, Sivén, Hall, Brugsch and Schittenhelm, Brugsch, etc.), while the minority come to the conclusion that the excretion of uric acid on a purine-free diet varies irregularly (Hindhede, Abl).

Some authors are of the opinion that the output of endogenous uric acid is constant for the individual when the diet is the same or nearly the same, but that the excretion is influenced by variations in diet (Umber, etc.).

The present report deals with the determination of uric acid in the urine and blood in seventeen individuals, two of whom were normal, while the remainder were convalescents and patients who had never had symptoms of gout. In addition to the quantity of uric acid the nitrogen output was determined in all the individuals, and in four the hydrogen ion concentration of the urine also was measured.

Method.—The uric acid concentration in blood was determined colorimetrically by Folin's method in the way previously de-

scribed (Höst, 1915, *a*). The method is correct within 10 per cent. The uric acid in urine was determined by the method of Folin and Denis, modified by Benedict. The nitrogen output was determined by the Kjeldahl method. The hydrogen ion concentration of the urine was determined at first by the electrometric method of Hasselbalch, and later by the colorimetric method, which I have already described (Höst, 1915, *b*).

In the present paper I shall report only the experiments with the two normal individuals.

EXPERIMENTAL.

Experiment 1.—Laboratory assistant and gardener, age 31 years, normal. The subject is a well built, healthy man and has never had symptoms of gout. During the investigations at the laboratory of Dr. Hindhede he maintained his weight on 4,200 calories. Throughout the experiments he therefore received this amount of calories, except in the underfeeding and overfeeding periods. The experiments were undertaken to study the influence of great variations in the amount of protein and calories in the diet on the output of endogenous uric acid. The experiment consisted of six periods.

Period 1.—May 8 to 11. Diet: coarse rye bread 1,200 gm. and butter 175 gm. (protein 98 gm., fat 163.5 gm., carbohydrate 600 gm.), equivalent to about 4,250 calories. The subject had lived on this diet since May 4, and thus the uric acid output even from the 1st day of the investigation was in proportion to the diet. On the 4 days of this period the uric acid output varied between 0.84 and 0.88 gm. (Fig. 1), and averaged 0.85 gm.

Period 2.—May 12 to 16. Diet: coarse rye bread 800 gm., butter 295 gm. (protein 67 gm., fat 269.5 gm., carbohydrate 400 gm.), equivalent to about 4,250 calories. In comparison with the first period this period contains about 30 per cent less protein and carbohydrate, while the fat was increased by about 60 gm., so that the caloric value remains the same. On the 1st day the nitrogen and uric acid output diminished considerably. The nitrogen continued to diminish slowly, while the uric acid excretion on the following 5 days had a constant value of 0.75 to 0.76 gm.

Period 3.—May 17 to 18. Diet: coarse rye bread 500 gm., butter 295 gm., dried apples 200 gm., sugar 50 gm. (protein 47.6

gm., fat 269 gm., carbohydrate 433.5 gm.), equivalent to about 4,300 calories. The protein was again reduced by 30 per cent, while the presence of carbohydrate was slightly raised and the fat remained the same. As the curve shows, both the nitrogen and the uric acid output diminished further on this diet. The uric acid excretion on the 1st day was 0.72 gm. and on the 2nd day 0.67 gm. Unfortunately the period was too short to show how much the excretion would have diminished on this diet.

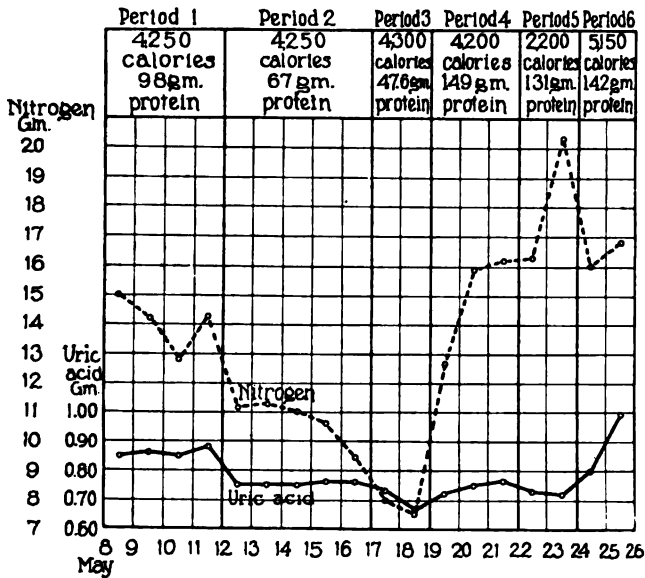


FIG. 1.

Period 4.—May 19 to 21. Diet: milk 1,000 cc., eggs 600 gm., butter 185 gm., and coarse rye bread 185 gm. (protein 149 gm., fat 264 gm., carbohydrate 301 gm.), equivalent to about 4,200 calories. The protein was then increased by about 200 per cent, while the carbohydrate was diminished 20 per cent, and the fat remained the same. On this protein-rich diet the nitrogen excretion immediately increased considerably, while the uric acid output grew slowly, and not before the 2nd and the 3rd days reached the amount of 0.75 and 0.76 gm., respectively (see Fig. 1).

Period 5.—Period 5, May 22 to 23, is one of underfeeding,

which consisted of milk 1,000 cc., eggs 600 gm., coarse rye bread 300 gm. (protein 131 gm., fat 96.5 gm., carbohydrate 185 gm.), equivalent to about 2,200 calories. The protein remained the same, while the fat and carbohydrate were diminished to such a degree, that the caloric value of the diet was lowered one-half. The nitrogen output on the 1st day was unchanged, but the next day it increased to 20.3 gm. Presumably the deficit in the food during the 1st day was made good by the glycogen in the liver, which acted to spare protein, so that the latter did not begin to be metabolized in increasing amount until the 2nd day.

Period 6.—May 24 to 25. Diet: coarse rye bread 1,500 gm., butter 200 gm. (protein 142 gm., fat 187.5 gm., carbohydrate 751 gm.), equivalent to about 5,150 calories. This period represents one of considerable overfeeding. The amount of protein was about the same as in the last period, but the quantity of the fat and especially that of the carbohydrate was much increased. Although the protein was a little increased, the nitrogen output on the 1st day diminished to about 16 gm., which was due to the fact that the organism, having abundance of nitrogen-free food-stuffs, economized with the protein metabolism. The uric acid output increased rapidly (Fig. 1) and the 2nd day reached a value of 0.99 gm. Unfortunately I had no opportunity to continue the investigations on this diet.

The excretion of endogenous uric acid is, in this subject, not constant because it varies considerably according to the composition of the diet as well as the quantity and quality.

During the 5 days of Period 2 the endogenous uric acid excretion was constant within the experimental error of the method. But on the last day of Period 1 the uric acid excretion increased considerably in spite of the fact that the diet was the same as on the former 3 days. The other periods were too short to determine whether the uric acid output was constant on the diet.

The influence of the composition of the diet on the uric acid excretion shows itself in: (a) Reduction of the protein and simultaneous increase of the fat and carbohydrate, total caloric value remaining constant, reduce the uric acid output. (b) Increase of the protein and simultaneous reduction of the carbohydrates in such a way that the caloric value is the same, increase the uric acid

output. (c) Underfeeding due to reduction of the nitrogen-free foodstuffs, the quantity of the protein being the same, effects a reduction of the uric acid excretion. (d) Overfeeding due to nitrogen-free foodstuffs, the protein being the same, increases the uric acid output.

From the results of the first four periods, it is seen that the uric acid output is not proportional to the quantity of the protein, the caloric value being the same. While in Period 1 the uric acid excretion is between 0.84 and 0.88 gm. and the protein of the diet is 98 gm., the uric acid output in Period 4 is only 0.75 to 0.76 gm. while the diet contains 149 gm. of protein.

When in Periods 1 and 4 the uric acid output is compared with the protein in the diet, it should be remembered that the protein in these two periods is given in different foodstuffs, in Period 1 essentially in bread, in Period 4 essentially in eggs and milk, and only to a lesser extent in bread.

The reduction of the protein in Period 1 is, as may be seen, due to a reduction of the quantity of the bread; it might therefore be doubtful, whether it is the bread itself or the protein in the bread that influences the uric acid output. That the protein as such is of importance is, however, evident from Period 4, in which the quantity of bread is the same as in Period 3, while the content of protein is much greater. The increase of the uric acid output in the fourth period is, however, not so large as might be expected from the amount of protein, and this fact suggests that the bread may have influenced the uric acid output. The great increase in the uric acid in Period 4 confirms this to some extent. This period represents one of overfeeding, which means increased uric acid output, but the increase the 2nd day is so great that the large quantity of bread, 1,500 gm., no doubt has been of importance in this connection. Unfortunately, I had no opportunity to continue the investigations on the influence of the coarse rye bread on the uric acid excretion.

The uric acid concentration in the blood was determined four times, with the following results:

22 Physiology of Endogenous Uric Acid

Date.	Uric acid concentration of blood.		
	I	II	Average.
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
May 10, 3 p.m.....	2.35	2.41	2.38
" 16, 3 "	2.51	2.60	2.60
" 18, 3 "	2.25	2.37	2.31
" 23, 3 "	2.20	2.34	2.28

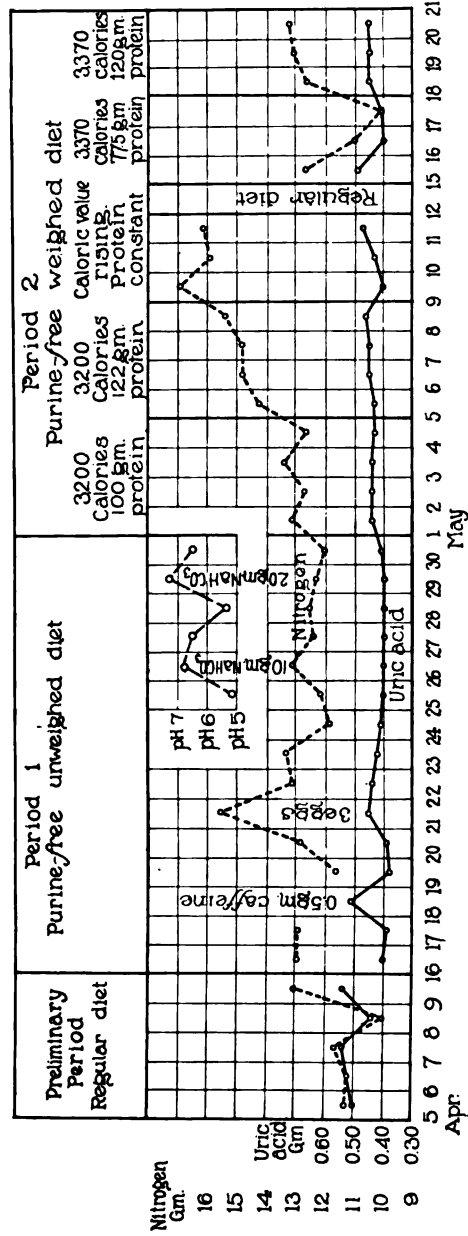
In spite of the fact that the uric acid excretion during 24 hours, as a result of changes in the composition of the food, varies greatly, the uric acid in the blood is constant within the experimental error of the method.

Experiment 2.—Physician, age 32 years, normal. The subject of the experiment has never had any serious illness or symptoms of gout. During the entire period of investigations laboratory work was carried on. The uric acid output was tested every 24 hours and in shorter periods. The experiment is divided into three periods.

Preliminary Period.—April 5 to 9. As is evident from Fig. 2 the uric acid output in the preliminary period is between 0.44 and 0.54 gm. each day. The nitrogen excretion shows in this period a complete accordance with the uric acid output.

Period 1.—In Period 1, April 10 to 30, in which the food was entirely purine-free, the uric acid output and the nitrogen output in the first 6 days were determined every 6 hours; namely, from 8 a.m. to 2 p.m., from 2 p.m. to 8 p.m., from 8 p.m. to 2 a.m., and from 2 a.m. to 8 a.m. The urine of the first 2 nights was collected from 8 p.m. to 8 a.m., but the results were with a view to correct proportions in the curve marked off as if divided in the periods 8 to 2 and 2 to 8.

It is evident from Fig. 3 that the uric acid output during the first 4 days is greatest between 8 a.m. and 2 p.m., less in the afternoon, and least at night. There seems to be no marked difference in the uric acid output before and after midnight. On the 14th some purine-containing food and tea and coffee were taken. The influence of this is seen immediately in the uric acid output, which is considerably increased in the night of the 14th to the 15th and in the forenoon of the 15th. The influ-



ence of the absorbed purines on the uric acid excretion was apparent until the afternoon of the 15th, that is 15 to 20 hours later. During the remainder of this period the urine was analyzed every 24 hours (Fig. 2). From the 16th to the 18th, the uric acid output was about 0.40 gm.; on the day 0.5 gm. of caffeine was given it increased to 0.51 gm., and the following day it decreased to about 0.40 gm. again. On the 21st the diet contained three eggs, in consequence of which the uric acid output immediately increased to 0.45 gm.; after this the excretion slowly diminished until on the 5th day it reached 0.40 gm. We observed the different way in which the uric acid output increased when caffeine and eggs were added to the food. During this period the influence of alkalies was also studied; on the 26th, and 29th, 10 and 20

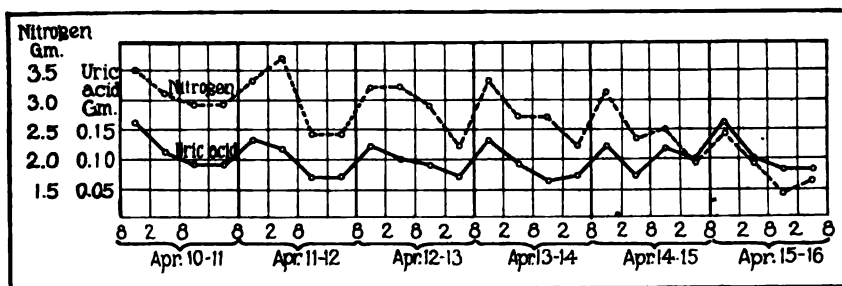


Fig. 3.

gm. of sodium bicarbonate, respectively, were given. The pH of urine increased, as Fig. 2 shows, to 6.80 and 7.30, respectively, but the uric acid excretion was not influenced in the least. Except for the increase of the excretion on account of caffeine and eggs, this period is characterized by a constant endogenous uric acid output at 0.40 gm.

Period 2.—In Period 2, from May 1 to 23, the diet consisted of weighed quantities of purine-free foodstuffs. In this period the influence of the composition of the diet and of the digestion on the uric acid excretion was tested more exactly. In the first 4 days the diet consisted of milk 1,000 cc., graham bread 450 gm., butter 100 gm., and two eggs (protein 100 gm., fat 167 gm., carbohydrate 321 gm.), equivalent to 3,200 calories. Upon beginning this diet the uric acid output increased to 0.44 gm. and remained

almost unchanged for all the 4 days. The cause of this increase cannot be determined with certainty since the composition and the quantity of the diet in the former were not constant. The subject of the experiment was of the opinion that the special quantity of bread and also that of the total amount of calories were increased in this period, which was probably the cause of the increased uric acid excretion. In the next 4 days of the period, May 5 to 8, the diet consisted of milk 1,000 cc., graham bread 375 gm., cheese 100 gm., butter 88 gm., and six eggs (protein 122 gm., fat 170.5 gm., and carbohydrate 284 gm.), equivalent to 3,200 calories. The quantity of calories was therefore the same, while the presence of protein increased from 100 to 122 gm., and that of the carbohydrate decreased correspondingly.

TABLE I.

Date.	Diet.	Protein.	Fat.	Carbo- hydrate.	Calories.
1915		gm.	gm.	gm.	
May 9	Bread 225 gm., butter 58 gm., cheese 135 gm., 6 eggs, milk 1,000 cc.....	111	145	260	2,670
" 10	Bread 410 gm., butter 115 gm., cheese 100 gm., 4 eggs, milk 1,000 cc.....	111	183	301	3,310
" 11	Bread 450 gm., butter 130 gm., cheese 135 gm., 2 eggs, milk 1,500 cc.....	121	208	362	3,835

The amount of fat was practically the same. Coincident with this change in the diet the uric acid output increased to about 0.45 gm. and during these 4 days averaged 9 mg. more than in the first 4 days. In the following 3 days, May 9 to 11, the amount of calories was varied by means of fat and carbohydrate, while the protein was about the same. The diet is given in Table I.

On the 1st day, May 9, the amount of calories was 2,670, an underfeeding which is at once accompanied by a decrease to 0.40 gm. in the uric acid output. The following 2 days the amount of calories increased to 3,310 and 3,835, respectively, and at the same time the uric acid excretion increased to 0.43 and 0.47 gm., respectively (Fig. 2). The increase of calories is due to a steady increase of fat and carbohydrate (Table I). The last day also the amount of protein rose slightly, from 111 to 121 gm., but this

26 Physiology of Endogenous Uric Acid

was too slight to explain the considerable increase of uric acid output from the 2nd to the 3rd day. From May 12 to 14 the experiments were interrupted; the diet on these days was regular and not purine-free. During the following 6 days, May 15 to 20, the relation between the uric acid output and the protein of the diet was studied by means of greater variation in the amount of protein than in Experiment 1. The diet is given in Table II.

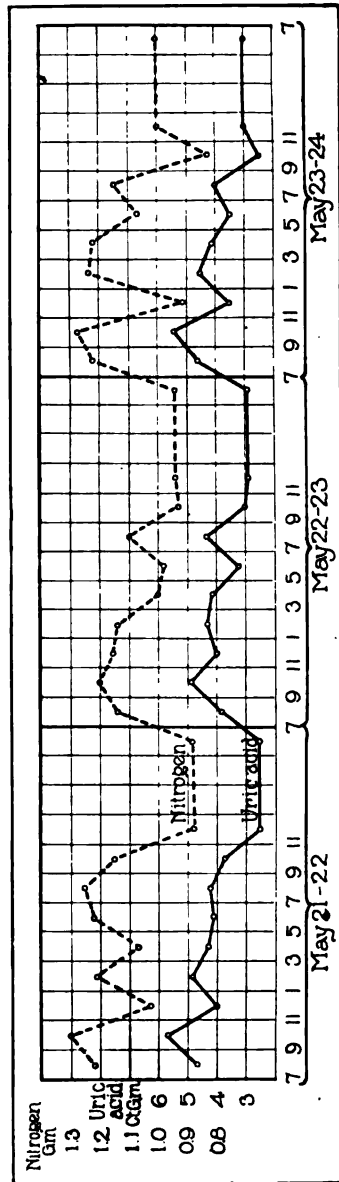
While in Experiment 1 a certain amount of carbohydrate was replaced with an isocaloric amount of protein, in Experiment 2 fat is replaced by corresponding quantities of protein, the amount of carbohydrate and calories being the same. On the first 3 days (May 15 to 17, Fig. 2) the diet contained 77.5 gm. of protein. The 1st day the uric acid output was rather great, doubtless owing to residual exogenous uric acid. The following 2 days the

TABLE II.

Date.	Diet.	Protein.	Fat.	Carbo- hydrate.	Calories.
1915		gm.	gm.	gm.	
May 15 to 17	Graham bread 450 gm., butter 200 gm., milk 1,000 cc.	77.5	220	276	3,372
" 18 " 20	Graham bread 450 gm., butter 136 gm., milk 1,000 cc., 6 eggs	120	198	276	3,370

uric acid excretion was 0.40 and 0.41 gm., respectively. From May 18 to 20 the amount of protein was increased to 120 gm. (i.e. about 50 per cent), and at the same time the uric acid output increased to 0.45 gm. and was exactly the same on the 2 following days.

During the last 3 days of the experiment, May 21 to 23, the diet was the same as on the preceding days, but from 7 a.m. to 11 p.m. the uric acid and the nitrogen output were determined every 2 hours in order to study the effect of the meals. The food was divided into three equal parts, taken at 7 a.m., 1 p.m., and 7 p.m. The uric acid output during the 3 days is parallel during each of the days (Fig. 4); the output is greatest in the morning and in the forenoon; it is slightly less during the remainder of the day and is least in the evening and at night. The daily output is given in detail in Fig. 4.



28 Physiology of Endogenous Uric Acid

The uric acid output was relatively great even between 7 and 9 a.m., and reached its maximum from 9 to 11 a.m., that is 2 to 4 hours after the first meal. From 11 a.m. to 1 p.m. the output decreased considerably, but increased again between 1 and 3 p.m. after the meal at 1. From 3 to 7 p.m. the excretion diminished, increased from 7 to 9 p.m. after the meal at 7, and decreased again at night.

Apart from the increases caused by the meals at 1 and 7 p.m. the curve is characterized by the fact that it rose sharply in the morning, reached its maximum early in the forenoon and then fell slowly and somewhat evenly down to a minimum at night. That the meal at 7 a.m. was not distinctly marked on the uric

TABLE III.

Date.	Uric acid concentration of blood.		
	I	II	Average.
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
<i>1915</i>			
Apr. 22, 5.30 p.m.....	1.94		1.94
" 26, 10.30 a.m.....	2.10	1.94	2.02
" 26, 5.30 "	1.94	1.94	1.94
May 3, 5.30 "	2.04	2.04	2.04
" 11, 5.30 "	1.92		1.92
" 16, 5.30 "	2.03	2.10	2.07
" 20, 12.00 m.....	2.00	2.12	2.06

acid curve, is undoubtedly due to the fact that the uric acid output had already begun to rise at this time of the day.

The uric acid concentration was determined seven times. The results are given in Table III from which it is evident that the endogenous uric acid concentration in the blood in this experiment was constant within the limits of error of the method, in spite of the fact that the food had been changed considerably both in quantity and quality, and the uric acid output had in consequence varied from 0.40 to 0.47 gm. in 24 hours.

The uric acid concentration of the blood was determined twice on the same day, before and 7 hours after the administration of 10 and 20 gm. of sodium bicarbonate, respectively. As is apparent from Fig. 2, the alkali taken produces just as little change in the amount of the uric acid in the blood as in the urine.

CONCLUSIONS.

From the results on the two normal individuals reported here and fifteen other subjects who were convalescents and patients the following conclusions are drawn.

In none of the seventeen subjects is the uric acid output for 24 hours constant. But with a fixed diet and under similar conditions the uric acid output in a few subjects was constant, while in the majority of those examined it was extremely irregular and showed variations from day to day up to 80 per cent.

But even in the individuals whose output of uric acid was most regular, the output was dependent on several factors, of which variations in the diet are the most important. In every increase or decrease of the caloric value of the food beyond a certain minimum, the uric acid output was always changed in the same direction. This took place whether the caloric value was varied by means of protein, fat, or carbohydrate; the change in the uric acid output is, however, greater when the amount of calories is varied by means of protein than by nitrogen-free food elements. With a constant food caloric value the uric acid output depended to a certain extent on the food protein, so that a change in the latter beyond a certain minimum always produced a corresponding change in the uric acid output.

The excretion of nitrogen and the hydrogen ion concentration of the urine were without influence on the endogenous uric acid output. On the other hand, increase of body temperature produced a considerable increase in the uric acid output.

The endogenous uric acid output in 24 hours varied between 0.27 and 0.99 gm.; the last value is obtained in a normal person on an extensive bread diet.

The amount of the endogenous uric acid in the blood varied to a great extent, the minimum being less than 0.50, the maximum being 2.68 gm. per 100 cc. of blood, but is in the case of each individual constant within the limit of error of the method (10 per cent). In 70 per cent of the subjects the uric acid concentration is between 0.01 and 0.02 gm. per 100 cc. of blood.

The investigations of previous workers show that the uric acid is not metabolized in the organism, and the experiments prove that the concentration of the endogenous uric acid in the blood

30 Physiology of Endogenous Uric Acid

in each individual is constant, the endogenous uric acid output becomes a direct expression for the uric acid formation, and we find the characteristic fact, that while in some individuals there is a regular endogenous uric acid production in others it is extremely irregular.

Definite conclusions regarding synthetic uric acid formation cannot be drawn from the experiments. The uric acid output and accordingly the uric acid production also depend to a certain extent on the quantity and quality of the purine-free diet. But the changes in the uric acid excretion, which are produced by variations in the food are, however, not so great that they may not be explained as depending on an increased or diminished operation on the part of the digestive organs. In this respect also the results of the uric acid determinations for the 2 hour periods agree, as they make it probable that a not inconsiderable part of the endogenous uric acid comes from metabolism in the tissue of the digestive glands.

It is characteristic of the endogenous uric acid metabolism that the concentration of uric acid in the blood is constant in the individual, but that it varies considerably in different individuals, and that the stability of the uric acid production varies greatly in different individuals. While some individuals produce and excrete daily a constant or almost constant amount of uric acid, the uric acid production in others is extremely irregular, so that the uric acid output in 24 hours is subject to relatively great variations.

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STUDIES ON THE BEHAVIOR OF INULIN IN THE ANIMAL BODY.

PRELIMINARY PAPER.

APPLICATION OF THE BENEDICT METHOD TO THE ESTIMATION OF LEVULOSE AND INULIN.

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(Received for publication, February 20, 1919.)

The experiments reported in the following paper were carried out by the author as a series of tests on the application of the Benedict (1) modification of the Lewis-Benedict method for sugar determination to the estimation of levulose and inulin, preliminary to a study of the behavior of the latter carbohydrate. Because these tests have made apparent the influence of certain factors which might easily be neglected in applications of this method to the analysis of *sugar solutions*, it has been considered worth while to publish the results.

Preparation of Standards.

The sample of glucose, analyzed by the gravimetric method of Munson and Walker, was 96.8 per cent pure; the levulose, by the method of Allihn, 98.2 per cent; while the samples of inulin used gave, after hydrolysis, reduction values equivalent to 94.7 and 90.5 per cent of that for the calculated amount of levulose; the moisture content of the samples was, however, 5.2 and 9.3 per cent.

Since pure picramic acid was not available, a sample of the acid assaying approximately 30 per cent of the estimated color was purified by recrystallization from boiling water according to the method of Egerer (2). With this product, the picramic acid standard, prepared according to Benedict's directions for the estimation of sugar in urine (1), was compared in a Duboscq colorim-

eter with the standard prepared by treating 1 mg. of the (96.8 per cent pure) glucose in 4 cc. of water with 4 cc. of the picrate-picric acid solution and 1 cc. of 20 per cent sodium carbonate, heating in a boiling water bath for 10 minutes, cooling, and diluting to 25 cc. When this glucose standard was set at 15 mm., the picramic acid matched it at 14.6 mm., or the picramic acid standard was almost exactly equal in color to a solution representing 1 mg. of 100 per cent pure glucose, treated as described above and diluted to 25 cc.

Estimation of Levulose.

When this picramic acid standard was set at 15 mm., 1 mg. samples of levulose, treated as in the preparation of the glucose standard described above, were found to match it at 14.9 to

TABLE I.
Estimation of Levulose in Presence of Inulin.
(Technique as for Glucose Standard.)

Weight of levulose added.	Weight of inulin added.	Time heated.*	Average reading.	Increase due to inulin.
mg.	mg.	min.	mm.	mm.
1.0	0.0	15	15.0	—
1.0	0.9	15	15.1	—
1.0	1.8	15	15.0	—
1.0	2.7	15	14.8	0.2
1.0	4.5	15	13.9	1.1

* After adding Na_2CO_3 .

15 mm. Since the levulose sample used was approximately 98.2 per cent pure, levulose must give a color very nearly equivalent to that from 102 per cent of its weight of glucose.

It was found, on experiment, that if quantities of inulin equivalent to three times the weight of sugar usually used for such a determination were heated with the alkaline picrate solution for 15 minutes, no deepening of the color produced by heating the picrate with the carbonate alone resulted; and also, as shown by Table I, that the addition of small quantities of inulin to the levulose samples caused no appreciable change in the color produced by the levulose in the alkaline picrate solutions. Hence, it seemed practicable to use this method for the estimation of levulose and of levulose in the presence of small amounts of inulin.

Effect of Heating the Sugars with Picric Acid.

The quantitative estimation of inulin by hydrolysis with the picrate-picric acid solution and determination of the levulose formed by heating it with sodium carbonate in the same solution was next attempted. This method was suggested by Rose's (3) determination of sucrose. Samples of inulin, 0.9 mg. each, were heated with the picrate-picric acid solution for periods varying from 10 minutes to 1 hour. To avoid the effect of the increased

TABLE II.
*Hydrolysis of Inulin by Picric Acid.**
(Readings Represent Duplicate Determinations.)

Date.	Weight of inulin.	Time heated, acid solution.†	Range of readings.	Average reading.	Inulin (Benedict, colorimetric.)	Inulin (Allihn, gravimetric.)
1918	mg.	min.	mm.	mm.	per cent	per cent
Nov. 11	1.0	10	17.3-17.5	17.4	77.5	90.5
" 11	1.0	15	16.2-16.4	16.3	82.8	90.5
" 11	1.0	20	15.5-15.8	15.7	85.9	90.5
" 7	0.9	25	16.1-16.2	16.15	92.8	90.5
" 7	0.9	30	15.7-15.8	15.75	95.1	90.5
" 7	0.9	35	15.1-15.3	15.2	98.5	90.5
" 13	0.9	20	15.1-15.2	15.15	98.6	94.5
" 13	9	30	13.1-13.2	13.15	114.4	94.5
1919						
Jan. 7*	0.9	40	16.4-16.9	16.65	90.9	94.5
" 7*	0.9	50	16.2-16.5	16.35	92.0	94.5
" 7*	0.9	60	16.0-16.2	16.1	93.7	94.5

* Determinations made with a sample of picric acid which had been purified by the method of Folin and Doisy; for the others commercial samples were used.

† Samples were, in every case, heated for 10 minutes after adding the sodium carbonate.

concentration of the picrate which resulted, the solutions were then cooled, made up to the original volume, the carbonate was added, and the tubes were again heated. As the results shown in Table II indicate, there was a deepening of the color developed on 10 minutes heating with the carbonate which increased with the time of heating in the acid picrate solution.

There seemed to be two plausible explanations for these results; namely, that the increased color was due either to the prolonged

action of the hot concentrated picric acid on the monosaccharide formed in the hydrolysis, or to the action of possible impurities in the picric acid used. A quantity of picric acid was, therefore, purified according to the method of Folin and Doisy (4), and a series of tests on the effect of heating glucose and levulose with the picrate-picric acid was made, each test being accompanied

TABLE III.

*Effect of Heating Glucose and Levulose with the Picrate-Picric Acid Solution.
(Readings Represent Duplicate Determinations.)*

Date.	Sugar.	Weight of sample.	Time heated, acid solution.*	Picric acid.	Average reading.	Control reading.†
1918		mg.	min.		mm.	mm.
Dec. 9	Glucose.	1.0	15	Purified.	14.7	15.2
" 11	"	1.0	20	"	14.4	15.2
Nov. 29	"	1.0	25	"	13.8	15.1
Dec. 9	"	1.0	30	"	13.3	15.2
Nov. 29	"	1.0	35	"	13.4	15.1
Dec. 9	"	1.0	45	"	13.3	15.2
Nov. 29	"	1.0	60	"	13.2	15.1
Dec. 9	"	1.0	60	"	13.1	15.2
" 13	"	1.0	60	"	13.2	15.2
" 11	"	1.0	60	"	13.2	15.2
" 12	"	1.0	60	Commercial.	13.2	15.2
" 13	"	1.0	70	"	12.5	15.2
Nov. 26	Levulose.	1.0	10	Purified.	14.3	15.1
" 26	"	1.0	20	"	13.4	15.1
" 26	"	1.0	40	"	13.3	15.1
Dec. 13	"	1.0	50	"	13.0	15.0
" 11	"	1.0	60	"	13.0	15.0
" 13	"	1.0	50	Commercial.	13.2	15.0

* Samples heated, in every case, for 10 minutes after adding the sodium carbonate.

† Control readings represent the readings of the same solutions of glucose and levulose which were heated only after adding the carbonate.

by a determination of the value of the sugar solution used according to the technique described for the preparation of the glucose standard (for detailed results see Table III). Since it was evident from these experiments that heating glucose and levulose with the picrate-picric acid solutions resulted in an increase in the depth of the final color varying with the time of heating in the

acid solution, the idea of estimating the value of inulin solutions by hydrolysis with the picrate-picric acid and colorimetric determination of the levulose in the same solution was abandoned.

Hydrolysis with Hydrochloric Acid.

Effect of Chlorides.

The next series of experiments dealt with the estimation of inulin by hydrolysis with hydrochloric acid, followed by a determination of the levulose by the Benedict method. To 90 mg. samples of inulin in 20 cc. of water was added HCl to give concentrations equivalent, respectively, to 0.5 and 1 per cent actual HCl. The resulting solutions were covered with watch-glasses to minimize evaporation, and heated over boiling water baths for varying lengths of time, heating for 1 hour with 0.5 per cent HCl being found to give the most nearly uniform results. The solutions were then cooled, neutralized exactly to methyl red with sodium hydroxide, made up to 100 cc., and 1 cc. portions analyzed according to the procedure recommended by Benedict for the preparation of the glucose standard for use in the determination of sugar in urine.

The results were so low, judging them from the analyses by the Allihn method, that their accuracy was questioned. The next step was, therefore, to study the effect of addition of HCl to solutions of glucose and levulose, using the same concentrations and technique as in the inulin determinations, and heating the solutions the usual 10 minutes for the development of the picramic acid color. It was evident that this procedure also gave results which were too low (Table IV).

In order to determine whether or not this effect was due to the destruction of the carbohydrate by the HCl, or to the interference of the NaCl formed by the neutralization of the acid with the development of the color, 20 cc. of the 1 per cent HCl were exactly neutralized with NaOH, made up to 100 cc., and amounts of this NaCl solution equivalent to those which had been present in the previous determinations were added to 1 mg. samples of glucose, and these analyzed in the usual way. The results were again low, indicating that the NaCl was in some way responsible. In order to eliminate the effect of possible impurities in the acid or alkali, a

TABLE IV.
Effect of NaCl, KCl, and HCl on Sugar Values as Determined by the Benedict Method.

Date.	Sugar.	Sample. mg.	Reagent added (1 cc. to 1 mg. sugar).	Time heated, acid solution.		Time heated, alkaline solution.	Picric acid.	No. of determi- nations.	Range of readings.		Average reading.	Control reading.*
				min.	min.				mm.	mm.		
Nov. 27, 1918	Glucose.	1.0	HCl†	60	10	10	Purified.	4	16.2-16.5	16.4	15.3	
" 27, "	"	1.0	HCl†	60	10	10	Commercial.	2	15.9-16.3	16.2	15.1	
" 29, "	"	1.0	NaCl†	—	10	10	Purified.	2	16.6-16.8	16.7	15.2	
Dec. 9, "	"	1.0	KCl§	—	10	10	"	2	16.4-16.5	16.4	15.2	
" 12, "	"	1.0	NaCl, 0.65%	—	10	10	"	9	16.8-17.3	17.1	15.2	
Jan. 13, 1919	"	1.0	NaCl, 0.65%	—	15	15	"	6	15.0-15.2	15.1	15.1	
" 15, "	"	1.0	NaCl, 0.65%	—	10	10	Commercial.	2	17.1-17.3	17.1	15.0	
" 3, "	"	1.0	HCl†	60	10	10	Purified.	2	16.4-16.7	16.6	15.0	
" 15, "	"	1.0	HCl†	60	10	10	"	4	17.0-17.4	17.1	15.2	
" 21, "	"	1.0	NaCl†	—	10	10	"	6	16.9-17.2	17.0	15.1	
Nov. 24, 1918	Levulose.	1.0	NaCl, 0.65%	—	15	15	"	4	14.9-15.4	15.2	15.2	
" 27, "	"	1.0	NaCl, 0.65%	—	10	10	"	4	14.9-15.4	15.2	15.2	
Dec. 11, "	"	1.0	NaCl†	—	10	10	"	4	14.9-15.4	15.2	15.2	
" 13, "	"	1.0	NaCl, 0.65%	—	10	10	"	4	14.9-15.4	15.2	15.2	
Jan. 15, 1919	"	1.0	NaCl, 0.65%	—	10	10	"	4	14.9-15.4	15.2	15.2	
" 15, "	"	1.0	NaCl, 0.65%	—	10	10	"	4	14.9-15.4	15.2	15.2	
" 27, "	"	1.0	NaCl, 0.65%	—	10	10	"	4	14.9-15.4	15.2	15.2	

* Control readings are those made on the same sugar solutions to which no salts had been added.

† 100 mg. of the sugar heated for 1 hour with 20 cc. of 1 per cent HCl, cooled, neutralized with NaOH, and made up to 100 cc.; 1 cc. portions taken for analysis.

‡ NaCl made by neutralizing 20 cc. of 1 per cent HCl with NaOH and making up to 100 cc.

§ KCl " " 20 " " 1 " " HCl " " KOH " " " " 100 "

|| Kahlbaum's zur Analyse NaCl.

concentration of Kahlbaum's *zur Analyse* NaCl corresponding to that formed by the neutralization of the acid, namely, 3.3 mg. per cc., was substituted. Again the results were the same.

These experiments were repeated between twenty-five and thirty times in order to find, if possible, some error in the technique. An interruption led to the increase in the time of heating the alkaline picrate solution on one day from the 10 minutes prescribed by Benedict to 15 minutes. It was found that in this case the colors of the solutions containing the NaCl exactly matched those of the control determinations, making it evident that, in some way, the effect of the NaCl was to retard the rate of development of the color; a difficulty which, as is indicated by Table IV, could easily be overcome by continuing the heating of the alkaline solution for 15 minutes. Determinations of inulin by HCl hydrolysis, followed by 15 minutes heating in the alkaline picrate solution, gave results which were comparative, but which were slightly lower than the total carbohydrate content of the samples as determined by the Allihn method.

Effect of Other Salts.

At this point an investigation of the effect of the presence of some of the other salts of the blood was undertaken. Solutions of 0.1 M disodium hydrogen phosphate, 0.1 M sodium dihydrogen phosphate, a buffer mixture of the two in concentrations corresponding to a hydrogen ion concentration of $\text{pH} = 7$, and a solution of NaCl representing a normal blood concentration of this salt (0.65 per cent NaCl) were made up. Determination of the value of 1 mg. samples of glucose to which 1 cc. portions of these solutions had been added were then made, using the technique described above for the preparation of the glucose standard. It was found that the dihydrogen phosphate tended to retard the rate of development of the color, while the disodium phosphate showed no effect at the end of 10 minutes heating, but, after 15 minutes, tended to give colors deeper than those of the glucose controls which contained no salts. That these effects might be due to changes in the hydrogen ion concentration seemed to be indicated by the fact that the buffer solution had no effect either on the rate of development of the color or the depth of the color

TABLE V.
Effect of Various Salts Normally Present in Blood on Determinations of Glucose* by the Benedict Method.

Date.	Sample.	Technique.	Added salt.	Time heated,† min.	No. of determinations.	Range of readings. mm.	Control reading. mm.
1919							
Jan. 20	mg.	As for glucose standard.	NaH_2PO_4 (1 cc. 0.1M)	10	4	16.9-17.1	15.2
" 22	1.0						
" 21	1.0	"	NaH_2PO_4 (1 cc. 0.1M)	15	2	15.0-15.1	15.1
" 20	1.0	"	Na_2HPO_4 (1 cc. 0.1M)	10	4	14.9-15.1	15.2
" 22	1.0						
" 21	1.0	"	Na_2HPO_4 (1 cc. 0.1M)	15	4	14.4-14.9	15.0
" 20	1.0	"	Buffer (1 cc. 0.1M)	10	4	15.2-15.4	15.2
" 22	1.0						
" 21	1.0	"	Buffer (1 cc. 0.1M)	15	2	15.1-15.1	15.1
" 20	1.0	"	Buffer + 0.65% NaCl (0.5 cc. each)	10	4	17.5-17.9	15.1
" 22	1.0						
" 27	4 mg. in 2 cc.	As for blood.	NaH_2PO_4 (2 cc. 0.1M)	10	2	15.0-15.1	15.2
" 27	4 " 2 "	"	Na_2HPO_4 (2 cc. 0.1M)	10	2	14.9-15.0	15.2
" 27	4 " 2 "	"	NaCl (2 cc. 0.65%)	10	2	15.0-15.0	15.2
" 27	4 " 2 "	"	NaCl (1 cc. 0.65%) + buffer (1cc { 0.1 M)	10	2	14.9-15.0	15.2

* Results of similar determinations of levulose are almost exactly identical with those for glucose.

† Time of heating after adding the sodium carbonate solution.

developed. Addition of 0.5 cc. of the buffer solution and 0.5 cc. of the NaCl solution to 1 mg. samples of glucose and levulose gave, repeatedly, a much greater retardation at the end of 10 minutes heating than 1 cc. of the NaCl alone, although 15 minutes heating was still sufficient for complete development of the color.

Effect of Adding Salts Directly to Blood.

The analyses reported above were made according to the procedure recommended by Benedict for the preparation of the glucose standard to be used in the determination of sugar in the urine. In order to ascertain the extent to which the factor of salt concentration might affect determinations of blood sugar, analyses of rabbit's blood, to which these salts had been added in concentrations of 1 cc. of the salt solutions described above to each cc. of blood, were made according to his procedure for blood. No effect of the salts was observed; the readings obtained after 10 and after 15 minutes heating with the salt solutions were exactly equal to those obtained with the blood alone, using the same technique. Addition of glucose and levulose to the blood samples resulted in their quantitative recovery, except that where the concentrations were considerably higher than in normal blood there seemed, after 15 minutes heating, to be a recovery of amounts slightly greater than those added. This may possibly be explained by some of the findings of Addis and Shevky (5), concerning the effect of prolonged heating on varying concentrations of sugar.

It was found also that values for the pure sugars as determined by the technique for blood, both after 10 and after 15 minutes heating of the sugar in the alkaline picrate solutions, were unchanged by the addition of these salts. It seems, therefore, that the larger concentration¹ of the picrate-picric acid solution called for by the method for the determination of blood sugar overcomes completely the effect of these salts.

¹ The method as applied to the determination of blood sugar involves the heating of 0.64 cc. of the sample equivalent approximately to 0.64 mg. of sugar, with $\frac{1}{16} \times 19$ cc. = 6.08 cc. of the picrate-picric acid solution minus the fraction used up in the precipitation of the proteins of the blood, as against the 4 cc. used for 1 mg. of glucose in the preparation of the standard for urine.

SUMMARY.

Benedict's modification of the Lewis-Benedict method has been used successfully for the determination of levulose, and of levulose in the presence of inulin.

When inulin is hydrolyzed by the picrate-picric acid solution, products are formed, which, on heating after the addition of the carbonate, give colors which increase in depth with the time of heating in the acid solution, and tend to become more intense than those from equivalent quantities of levulose. The color developed by glucose and levulose solutions which have been heated with the acid picrate before the addition of the carbonate is also more intense than that given by the same amounts of the sugar solutions treated by the usual procedure.

When the quantities of the picrate-picric acid solution recommended by Benedict for the preparation of the glucose standard for the determination of sugar in urine are used, the addition of small amounts of sodium chloride and sodium dihydrogen phosphate to samples of glucose and levulose cause very appreciable retardation in the rate of color development. Therefore, when it is desired to estimate the monosaccharide formed by acid hydrolysis of a more complex carbohydrate, the influence of the salt formed by the neutralization of the acid must be taken into consideration. Interference of these salts may be avoided by increasing the time of heating with the carbonate to 15 minutes or by using the higher concentration of the picrate-picric acid called for in the determination of blood sugar.

The writer is indebted to Professor H. B. Lewis of this laboratory for helpful suggestions and criticisms.

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DIGESTIBILITY OF BACON.

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(Received for publication, March 19, 1919.)

Rubner,¹ 40 years ago, published figures for the digestibility of bacon fat which showed low utilization, a loss in the feces of 17.4 per cent of the fat eaten. This is so different from figures published then and since,^{2,3} for the utilization of soft fats including lard, and so different also from the every day experience of the satisfactory character of bacon as food, that it seemed desirable to perform other digestibility experiments with bacon. Rubner's own explanation of the extensive loss was that the bacon was eaten in large lumps and the fat was imbedded in connective tissue. It may be also that in his experiment bacon was eaten raw, though he does not say so. He found large pieces of fat in the feces, not disintegrated.

Our experiment was carried out in the spring of 1916 by three young women⁴ students in two periods of 3 days each, with an interval of 3 days in which ordinary food was eaten. Three other students later took part in a shorter supplementary experiment.

The diet consisted of bacon, shredded wheat biscuit, orange juice, and a small amount of sugar. The bacon, obtained through the courtesy of Swift and Company, was specially selected in order to be as nearly uniform as possible and to have a large percentage of fat. In Period 1 it was cooked only slightly and in

¹ Rubner, M., *Z. Biol.*, 1879, xv, 170. Quoted by von Noorden, C., *Metabolism and practical medicine*, 1907, i, 55.

² Cf. Langworthy, C. F., and Holmes, A. D., *U. S. Dept. Agric., Bull.* 310, 1915.

³ Cf. Smith, C. A., Miller, R. J., and Hawk, P. B., *J. Biol. Chem.*, 1915, xxiii, 505.

⁴ The careful cooperation of all these students is gratefully acknowledged, especially Miss Mary Rising who did a part of the analytical work.

Period 2 much more thoroughly. The average raw weight eaten per day was 350 gm. during Period 1 and 790 gm. during Period 2. Since the slightly cooked bacon lost on the average 66 per cent of its weight in cooking and the much cooked 80 per cent, the cooked bacon eaten averaged 119 gm. and 158 gm. for the two periods, giving 83 and 102 gm. of fat. In preparing the thoroughly cooked material the process was continued till the steam had almost ceased bubbling off, but no charring had commenced.

To obtain the composition of the cooked bacon a number of analyses for fat and nitrogen were made on similar samples. Fat was determined as usual by ether extraction, and nitrogen by Kjeldahl on the fat-free residue. Close duplicates could not be obtained on the somewhat variable material, especially for the

TABLE I.
Composition of Cooked Bacon.

	Loss of wt. in cooking.	Fat.	Nitrogen.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Slightly cooked.	56-58	74.5	2.5
	65-69	71.5	3.2
Much cooked.	79	64.5	4.5
	82	61.9	4.8
	87	60.3	4.7

slightly cooked, but since twelve different determinations were made on it the resulting figures should be reliable. The averages are given in Table I and on two curves plotted in Fig. 1. To find the amount of bacon fat consumed at each meal the loss of weight in the cooking was noted and the corresponding percentages of fat were read from the curve.

The small amount of fat in shredded wheat biscuits was computed from published analyses,⁵ since they seemed sufficiently uniform (1.4 per cent). As the nitrogen from this source was a somewhat large proportion of the nitrogen of the diet, it was determined as usual and found to be 1.73 per cent. The orange juice nitrogen

⁵ U. S. Dept. Agric., *Off. Exp. Stat. Bull.* 28, 1906; *Farmers' Bull.* 249, 1906; *Modern Hospital*, 1915, iv, 165.

was taken as 0.09 per cent.⁶ The amount and composition of food eaten are given in Table II.

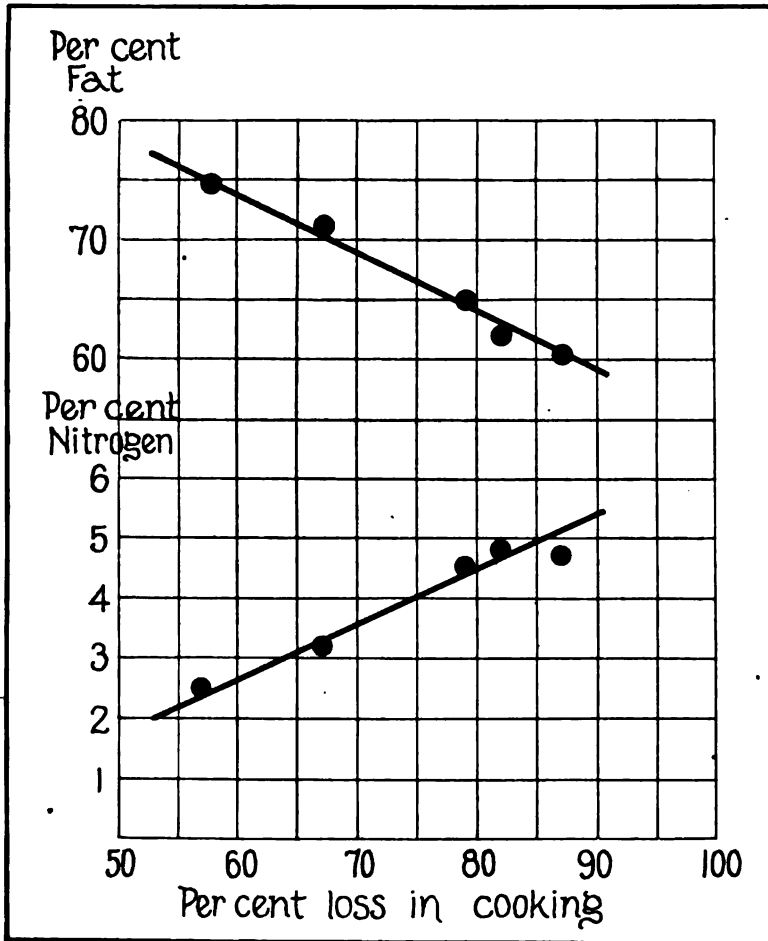


FIG. 1. Curves of composition of cooked bacon.

To identify the feces of the test period charcoal was taken at the first and last meal. Daily collection of the feces was made

⁶ Thorpe, E., Dictionary of applied chemistry, London and New York, 2nd edition, 1913, iv, 21.

Digestibility of Bacon

TABLE II.

Food.

	Subject M.			Subject B.			Subject R.		
	Quantity.	Fat.	Nitrogen.	Quantity.	Fat.	Nitrogen.	Quantity.	Fat.	Nitrogen.
		gm.	gm.		gm.	gm.		gm.	gm.
Cooked bacon...	355 gm.	261	11.9	348 gm.	235	11.0			
Shredded wheat.	390 "	5	6.8	490 "	7	8.6			
Orange juice.....	1,080 cc.		0.9	1,080 cc.		0.9			
Total.....		266	19.6		242	20.5			
Cooked bacon...	493 gm.	316	21.8	498 gm.	322	21.4	435 gm.	283	19.2
Shredded wheat.	390 "	5	6.8	364 "	5	6.3	468 "	6	8.1
Orange juice.....	1,265 cc.		1.1	1,450 cc.		1.3	1,407 cc.		1.2
Total.....		321	29.7		327	29.0		289	28.5

TABLE III.

Feces.

	Subject M.					Subject B.					Subject R.				
	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.	Wt. of stool.	Total fat.	Fatty acid.	Neutral fat.	Nitrogen.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Period 1.	94	7.1	3.0	4.1	1.39	49	3.1	1.4	1.7	0.60					
	60	3.6	1.1	2.5	0.59	149	8.6	3.7	4.9	1.80					
	108	5.0	2.5	2.5	1.55	67	4.4	2.1	2.3	0.77					
	27	1.4	0.6	0.8	0.48	61	3.1	1.2	1.9	0.85					
Total.....		17.1	7.2	9.9	4.01		19.2	8.4	10.8	4.02					
Period 2.	47	3.0	1.0	2.0	0.88	30	1.8	0.6	1.2	0.80	170	8.6	5.1	3.5	2.80
	114	4.4	1.7	2.7	1.86	158	6.1	2.2	3.9	3.22	92	2.9	0.9	2.0	1.19
	95	5.5	1.9	3.6	1.91	131	5.3	2.2	3.1	1.94	131	5.6	2.5	3.1	2.01
						39	2.5	1.0	1.5	1.04					
Total.....		12.9	4.6	8.3	4.65		15.7	6.0	9.7	7.00		17.1	8.5	8.6	6.00

from the time of the first to the last appearance of the dark coloration. Daily feces were weighed, thoroughly mixed, and sampled for analysis. The analyses were made on a wet basis. The Saxon method⁷ was used for the determination of fat. According to this, the moist sample is treated with water, hydrochloric acid, alcohol, and ether, the top ethereal layer containing the fat and the fatty acid repeatedly blown off, the ether evaporated, and the fat weighed. The fat was then titrated with 0.05 N sodium hydroxide to determine the free fatty acid of the feces, as was done by Smith, Miller, and Hawk.⁸ The fatty acid represents the fat which has been acted upon by the enzyme but not absorbed; i.e., digested but not utilized. The total fat represents the fatty acid and the

TABLE IV.
Summary. Coefficients of Utilization.

	Subject M.		Subject B.		Subject R.	
	Period.		Period.		Period.	
	1	2	1	2	1	2
Fat eaten, gm.....	266	321	242	327		290
Total fat in feces, gm.....	17.1	12.9	19.2	15.7		17.1
Neutral fat, gm.....	9.9	8.3	10.8	9.7		8.5
Fat digested (split), per cent.....	96.1	96.4	96.6	96.0		96.8
“ utilized, per cent.....	93.6	95.9	92.1	95.1		94.1
Nitrogen eaten, gm.....	19.3	29.7	20.6	29.0		29.4
“ in feces, gm.....	4.01	4.65	4.02	7.00		6.00
“ utilized, per cent.....	79.2	84.4	80.4	75.8		79.7

unsplit or neutral fat. Results for fat and nitrogen are given in Table III. The percentage of total fat occurring as free fatty acid is about the same in this experiment as in that of Smith, Miller, and Hawk,⁸ theirs being 41.6 per cent and 42.4 per cent in our experiment.

The average percentage of digestion of the fat of slightly cooked bacon was 96.3, and of the much cooked 97.0 (Table IV); while the average percentage of the utilization of the slightly cooked bacon was 92.8, and of the much cooked 95.0, approximately the same as the figures for most fats given by Hawk, by Langworthy

⁷Saxon, G. J., *J. Biol. Chem.*, 1914, xvii, 99.

and Holmes, and other investigators. There is little difference between the two ways of cooking. Especially noteworthy, however, is the fact that our utilization of bacon fat is much higher than Rubner's, who found the fat utilization to be only 82.6 per cent. As mentioned above, he speaks of finding unchanged pieces of bacon in the feces. In our experiment very minute particles were found, which were apparently unchanged shredded wheat but not fat.

These results were confirmed later by a single 3 day experiment in which three students ate the same diet, using moderately cooked bacon and determining the fat only in the mixed feces of the period. The coefficients of utilization were 96.2, 95.7, and 96.5 per cent.

The nitrogen of the diet was not so well utilized—79.8 per cent on the average in Period 1 and 80.0 per cent in Period 2. These low figures, however, are undoubtedly due not to the bacon nitrogen but to the shredded wheat. In the diet of Subject M, for example, in Period 1, 34.7 per cent of the nitrogen came from the shredded wheat, the coefficient of digestibility of which has been reported⁸ as only 57.7 per cent. Only 65.3 per cent of the nitrogen came from the meat and the small amount of orange juice. A computation, $79.8 - (34.7 \times 0.577) = X \times 65.3$, shows that X , or the digestibility of the bacon and orange juice nitrogen, is 92 per cent. This is not far from the usual figure for meat protein.

SUMMARY.

The coefficient of digestibility of much and slightly cooked bacon fat is found to average 96.7 per cent, approximately the same as that for other soft fat and much higher than the 82.6 per cent reported by Rubner for bacon eaten in large pieces (raw?). The nitrogen also is as well digested as that of other meat.

⁸ Maine Agricultural Experiment Station quoted by Street, J. P., *Modern Hospital*, 1915, iv, 165.

COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION IN SMALL QUANTITIES OF SOLUTION.*

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(Received for publication, March 3, 1919.)

The hydrogen ion concentration (1) of many solutions and of culture media is readily determined by means of buffer solutions and suitable indicators. The usual procedure, in determining colorimetrically the hydrogen ion concentration, requires a considerable volume of solution. Frequently the quantity of solution available in experiments upon plants or animals is extremely limited in amount. It is of interest, therefore, to ascertain which modifications of the colorimetric method are best suited for the determination of the hydrogen ion concentration in small volumes of solution.

Recently, several such modifications have been used by different investigators. In determining the pH values of small quantities of plant sap, Wagner (2) has made use of lacmosol (3) as an indicator in a microchemical method. The indicator was found to give excellent results between pH 4.2 to 6.0. A very small quantity of the sap was placed on a slide to which was added the same quantity of lacmosol as had been added to a series of slides each bearing buffer solutions of known pH value. The concentration of the indicator was the same for each slide. The matching of the colors of the indicator was at first carried out by means of the spectroscope. The use of the spectroscope was found so laborious that the colors were compared preferably by the use of two microscopes with twenty magnifications, whereby differences as small as 0.03 in pH could be detected.

The spectroscope has been used by Tingle (4) to determine the neutral point (in titrations for total acid) in solutions in which the natural color of the solution masks the color of the indicator. In such cases, much larger quantities of indicator are required than would be desirable in ordinary

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titrations. The conclusion is reached, save in the case of methyl orange, that it is inadvisable to use the spectroscope for titrations except when the liquid is so colored that no other means can be used for detecting the end-point.

In determining the hydrogen ion concentration of the sap of succulents, Miss Hemple (5) has found it impossible in many cases to employ the colorimetric method as drawn up by Sørensen (6) owing to the color present in the sap. Accordingly, two forms of the electrometric method (7, 8) were employed by her as an accurate means of determining the pH value. However great the exactness of these methods, it was frequently found advisable to employ a method whereby it was possible in the course of the investigation to ascertain rapidly and without waste of material the condition of the sap of a plant at any interval of time. At the suggestion of Professor Sørensen she examined various indicator papers as produced at the Carlsberg laboratory and found that lacmoid paper with a range of pH 3.8 to 6.0 was suitable for the purposes of the experiment at hand. A drop of the unknown solution was applied to a piece of lacmoid paper and the color produced upon the paper was then matched with the colors produced by drops of buffer solutions of known hydrogen ion concentration.

In comparing the electrometric with the lacmoid paper values for the pH of the sap of succulents, Hemple found that rarely did a divergence of 0.4 to 0.5 in pH occur between both methods and that the difference was generally only 0.2 to 0.1.

The use of lacmoid paper, while it does not give an exact means of measuring the hydrogen ion concentration, does afford a rapid and convenient method of obtaining an approximate value. I have found that frequently in bacteriological, as well as in plant physiological work, it is desirable to know quickly and without waste or contamination of a solution, the approximate hydrogen ion concentration. Accordingly the various indicators that are now most widely used in colorimetric determinations were investigated with the view of obtaining indicator papers that would cover the usual working range of pH.

It was soon found that many of the sulfonephthalein (1) group of indicators, that give such excellent results when used in solutions, do not lend themselves readily for the preparation of stable indicator papers unless extreme precautions are taken to keep them in an atmosphere free from carbon dioxide and to protect them as much as possible from the light. Indicator papers, prepared with bromocresol purple, bromothymol blue, phenol red, phenolphthalein, or with mixtures (1) of these, may, under ordinary conditions, even by their evanescence or rather reversibility of

color change, serve to give us a clue as to the approximate range in which the hydrogen ion concentration value lies.

Lacmoid paper was prepared according to the method described by Glaser (9). Varying results are obtained according to the care taken in the preparation of the paper. A small quantity of Mercks lacmoid was digested with 96 per cent alcohol on a water bath and the solution was then filtered. The alcoholic filtrate was used directly in the preparation of the indicator papers. If it is desired, the filtrate may be evaporated to dryness in a vacuum at a low temperature. It was thought best to prepare both the red and the blue papers in order to read the more acid end of the range on the blue paper and the less acid end of the range on the red paper. Various intermediate colored papers were also prepared.

To prepare the blue lacmoid paper, dilute sulfuric acid was added to the alcoholic lacmoid solution until the strips of white filter paper were no longer colored blue but became red. If too much acid has not been added, the paper becomes blue on drying. The sulfuric acid should be added carefully and test papers made upon each small addition of acid in order to add just the correct amount of the acid.

To prepare the red lacmoid paper, dilute sulfuric acid was added to the alcoholic solution of the purified lacmoid until the test papers made from the solution appeared rose-red after several hours of drying. If too much acid has been added, then the sensitivity of the paper is decreased.

The papers were suspended upon strings. They usually require to be drawn a second time through the indicator solution, once they have become dry, in order to give the paper an even tone of color. The drying should not be hastened and should be carried on preferably in a carbon dioxide-free atmosphere over soda lime. The depth of color selected depends upon the ability of the investigator to distinguish color differences, and, as a rule, the lighter shades appear to be preferable to the darker shades. The success in preparing sensitive indicator paper depends also to a large degree on the quality of filter paper selected. Strips of a good grade of quantitative filter paper about 1 cm. in width are very convenient. The ordinary filter paper, that has a mottled appearance due to depressions in its surface, cannot be depended

upon to give uniform results. It is advisable to prepare trial indicator papers from several lacmoid solutions that give the red or the blue indicator papers in order to be able to make a selection for sensitivity.

The method of using indicator paper seems to be of sufficient importance to warrant explanation. The procedure followed by us has been to lay, with forceps, the indicator paper upon a piece of filter paper and then to allow a drop of the solution, the pH of which is to be determined, to fall upon the indicator paper. In a similar manner, once the range of pH has been approximately located, a set of standards is prepared by allowing a drop of buffer solutions of known pH to fall upon the indicator paper strips. The strips are immediately placed in a carbon dioxide-free atmosphere over soda lime to dry slowly. The comparison of the color caused by the solution of unknown pH with the colors caused by the buffer solutions is made best when the papers have dried, though it is advisable to observe the papers at intervals in order to be aware of undue changes in color, should any occur. The reading or comparison of colors is made best away from the central part of the drop and not too close to the edge of color change, as here the diffusion processes may give incorrect results. By coating the dried papers with a thin layer of a good grade of paraffin, free from appreciable amounts of acid, more permanent standards may be secured.

With a view toward completing the more acid end of the pH scale, methyl orange paper was prepared with a range of pH 2.4 to 3.8. Strips of a good grade of quantitative filter paper were drawn through a filtered aqueous solution of the indicator and dried on strings. The lighter yellow shades of the indicator paper were preferable to the darker shades as the color changes were the more easily distinguishable.

Bromophenol blue indicator paper with a range of pH 3.4 to 4.6 was prepared by drawing the strips of filter paper through the indicator solution (1) until a uniform blue color was obtained:

Alizarin sodium sulfonate indicator paper with a range of pH 4.0 to 6.0 was prepared by dissolving the indicator in pure distilled water containing a small quantity of alcohol and then proceeding with the strips of filter paper as previously described. The sensitivity of the indicator paper may be somewhat increased by a small addition of dilute sulfuric acid.

An aqueous solution of neutral red pH 7 to 9 may be used to prepare indicator paper for this range, though other papers for this range are much needed.

Azolitmin indicator paper serves to cover the range of pH 6.2 to 8.0. Much of the criticism recently directed against the use of indicator papers has been in connection with the use of litmus in determining the degree of soil acidity. The sensitivity of various blue litmus papers of commerce for the determination of soil acidity has been tested by various investigators (10, 11), with the result that litmus paper determinations of soil acidity are considered unreliable unless unusual precautions are taken.

The writer has found that when the soil is extracted with as little water as possible (12) in order not to dilute the buffer substances more than necessary, and the soil extract is centrifuged free from most of the colloidal material, that the drop method upon azolitmin paper gives a fairly close approximation to the pH value as obtained by the colorimetric method of Sørensen.

Unfortunately much of the litmus paper of commerce is prepared from litmus products of varying degrees of purity, and the product may differ according to the method followed in obtaining it. The litmus of commerce contains, on an average, about 4 to 5 per cent of azolitmin.

A Merck's preparation of azolitmin was dissolved in pure distilled water containing a very small quantity of dilute sodium hydroxide and then the color was adjusted to the shades of the indicator paper desired by successive small additions of dilute oxalic acid.

The concentration of the indicator solutions used in the preparation of the sulfonephthalein papers was approximately of the strength described by Clark and Lubs (1). The other indicator solutions were prepared by diluting concentrated solutions of the indicators until strips of the filter paper gave the desired tint.

For this purpose, strips of indicator paper were prepared from the concentrated solutions and tested. In this manner the dilutions were continued until the dried indicator papers from the indicator solution showed the highest sensitivity when tested with buffer solutions of known pH value.

The perfection of a suitable series of indicator paper standards awaits only the development of the possibilities of the ever growing series (13) of useful indicators (14) now at our disposal.

We have found it convenient, as well as advisable, when dealing with very small volumes of solutions that have been tested by the indicator paper method, to have a check upon the obtained pH values in another way. Such a method consists in placing one or more drops of the solution, whose pH value is being tested, in a cavity of a porcelain spot plate and by placing in other cavities of the plate the same quantity of buffer solutions of known pH value, and then adding the same concentration of indicator in each cavity. The indicator may be diluted on the spot plate and is conveniently added to the small quantities of solution by means of a platinum loop which can be used as a stirring agent, or the diluted indicator can be added from a dropping bottle and the platinum loop used to mix the indicator with the solution. The indicators of Clark and Lubs (1) are most excellent for this method. The color of the solution that is being tested is then compared with the colors developed in the buffer solutions of known pH value. It is understood, however, that whenever enough solution is available and the greatest degree of accuracy is desired, the usual Sørensen colorimetric method and preferably the electrometric method should be used.

SUMMARY.

The indicator paper method, when used with precaution, because of its rapidity and the small volumes of solution required in testing for the pH value, deserves greater use than it has received in the past. The supplementary use of the spot plate method adds to the reliability of the indicator paper determinations.

Although the electrometric or the usual Sørensen colorimetric methods are recognized as being superior, there are cases in which such refined methods either cannot be applied or require too much time. With the described methods it is usually possible to obtain a pH value differing 0.4 to 0.2 pH or even less from those obtained by the more exact methods.

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A RAPID METHOD FOR THE ESTIMATION OF UREA IN URINE.

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WITH THE ASSISTANCE OF AARON BODANSKY.

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(Received for publication, March 26, 1919.)

The method, which is an adaptation of a procedure used in this laboratory for testing the strength of urease preparations, consists in treating a small quantity of urine in a centrifuge tube with an approximately neutral phosphate solution and with urease. After the urea has been decomposed a protein precipitant is added, the tube centrifuged, and an aliquot of the clear supernatant liquid is Nesslerized and compared with a standard.

The amount of urine chosen was 1 cc., and the phosphate solution made of such strength that 1 cc. of it would neutralize the ammonia formed from 1 cc. of concentrated urine.

We have used all manner of urease preparations and have come to the conclusion that finely ground jack bean powder is the most satisfactory, since it is easily prepared and keeps well. Moreover, the powder prepared in this laboratory contains no ammonia and forms none unless subjected to the action of alkali, as in the Marshall-Van Slyke-Cullen aeration method.¹

The powder has the disadvantage of being difficult to measure out, but if moistened with alcohol it can be moulded readily into tablets which possess enough coherence for handling, but which disintegrate rapidly when added to an analysis.

To hasten the action of the enzyme the test is warmed momentarily to about 50°C., either by shaking over a small flame or by placing in a water bath, and is then allowed to cool for 20 minutes so that it will reach room temperature before the aliquot is taken.

¹ Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 493. Marshall, E. K., Jr., and Davis, D. M., *ibid.*, 1914, xviii, 53. Van Slyke, D. D., and Cullen, G. E., *ibid.*, 1914, xix, 211; 1916, xxiv, 117.

During the warming no appreciable amount of evaporation will take place unless the material is shaken to the upper part of the centrifuge tube, but some slight evaporation will occur during the centrifuging. To avoid this chance for error, and to make it easier to mix the contents after adding the protein precipitant, tightly fitting cork stoppers which have been soaked in beeswax are used. Care must be taken to cut off the tops of these stoppers, if necessary, so that they will not strike the centrifuge head while in motion.

The precipitant used is acidified potassiomeric iodide. This forms a precipitate which settles very quickly on centrifuging, and this reagent is the most logical to use where the solution is to be Nesslerized afterwards.

Acidified potassiomeric iodide solution liberates iodine on standing, but this decomposition can be prevented by the addition of a small amount of sodium bisulfite. Unless the solution is kept tightly stoppered the bisulfite will have to be added from time to time. Whenever free iodine is present it will be plainly indicated by its action on the starch in the urease tablets.

After adding the potassiomeric iodide the solution must be mixed, which is done by placing the thumb on the stopper and inverting. After mixing, the stopper should be removed for an instant to allow the carbon dioxide liberated by the acid to escape. Centrifuging will usually take about 1 minute. After centrifuging, if it is desired, the analysis can be left for several days before completion, if tightly stoppered. The amount of supernatant liquid most likely to yield 1 mg. of ammonia nitrogen is 1 cc., but if the Nesslerized solution is too dark or too light there will be enough liquid left in the centrifuge tube for another Nesslerization.

Centrifuge tubes that have been used in an analysis are washed with a small test-tube brush covered with soap, and are then rinsed with a solution of potassium iodide and sodium hydroxide to remove the last traces of mercury. *This is absolutely essential*, otherwise the enzyme will be poisoned when the tube is next used for an analysis. The stoppers need only to be rinsed in water and dried with a towel after use, as the enzyme does not come in contact with them during its action. Pipettes that have been in contact with solutions containing mercury must be rinsed with the alkaline potassium iodide before being used to pipette urine or phosphate solution.

Preparation of Reagents.

Phosphate Solution.—Dissolve 111 gm. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 85 gm. of KH_2PO_4 in about 400 cc. of warm water and dilute to a volume of 500 cc. The solution can be conveniently kept in a bottle fitted with a stopper through which passes a 1 cc. Ostwald pipette, dipping into the liquid not more than 1 or 2 cm. This pipette, as well as others used in the analysis, should be calibrated.

Urease Tablets.—Grind about 50 gm. of jack beans, first in a coffee mill and afterward in a large porcelain mortar, until nearly as fine as wheat flour. Sift gently through cheese-cloth to remove the coarser particles. Moisten the sifted material with a small amount of 95 per cent alcohol and immediately press into a hard rubber tablet mould for making 1 grain tablets. After smoothing and pressing out, the tablets are allowed to dry for several days and are kept in a stoppered test-tube. Two of these tablets, the amount used for an analysis, will weigh about 0.1 gm. and form approximately 11 mg. of ammonia nitrogen in 5 minutes at 20°C . when treated with an excess of phosphate-urea solution. The most practical way, however, of testing their enzyme strength is by the regular method for the analysis of urea in urine described in this paper, using a concentrated sample of urine, and allowing the enzyme to act for 10, 15, and 20 minute intervals after the preliminary heating. The results are then compared. Urease tablets prepared in this laboratory have been found to require 15 minutes plus the preliminary heating to hydrolyze the urea in 1 cc. of urine completely, but to be on the safe side the time that the enzyme is allowed to act has been lengthened to 20 minutes.

Acidified Potassiomericuric Iodide Solution.—To 100 cc. of the potassiomericuric iodide solution of Folin and Denis² add 50 cc. of concentrated hydrochloric acid, dilute to 1 liter volume, add 1 gm. of sodium bisulfite, and keep in a stoppered bottle fitted with an accurate 3 cc. pipette.

² Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 479.

Procedure.

Into a clean, dry 15 cc. centrifuge tube, which is not sharply constricted, carefully pipette 1 cc. of urine³ and 1 cc. of the phosphate solution, using Ostwald pipettes. Add two urease tablets, stopper, and shake gently over a small Bunsen flame until the contents of the tube feel just uncomfortably hot to the hand (or warm by allowing the tube to stand for 3 or 4 minutes in water heated to 55°C.). The material should become well mixed during this heating. Allow the tube to stand in a rack for 20 minutes. Now add exactly 3 cc. of the acid potassiomeric iodide solution, stopper tightly, invert, and shake violently to mix. Remove the stopper for an instant to relieve the gas pressure and centrifuge for 1 or 2 minutes. According to the concentration of the urine used, pipette 0.5, 1, or 2 cc. of the supernatant liquid into a 100 cc. volumetric flask, dilute, Nesslerize with 15 cc. of the Nessler solution of Folin and Denis,² make up to volume, and compare in a colorimeter with a 1 mg. standard Nesslerized at the same time. The reading of the standard divided by the reading of the unknown and multiplied by 10, 5, or 2.5, according to whether 0.5, 1, or 2 cc. have been taken as the aliquot, will give the urea and ammonia nitrogen in the original urine in mg. per cc. or gm. per liter. The values for the urea alone can be calculated by subtracting the corresponding figures for ammonia.⁴

Blanks.—To be certain that the method is being properly carried out it may be well for the analyst to run an experiment using, instead of 1 cc. of urine, 2 cc. of an ammonium sulfate solution containing exactly 2 mg. of nitrogen. After adding the reagents and centrifuging, 3 cc. of the supernatant liquid are Nesslerized to 100 cc. volume and compared in the colorimeter with the usual 1 mg. standard. The reading of the blank should agree with the reading of the standard to within about 1 per cent.

To make certain that the reagents contain no ammonia a blank is run, using distilled water instead of urine. Approximately neu-

³ If the urine is extremely concentrated it will be advisable to use only 0.5 cc., bearing in mind that the total volume of the analysis in this case will be 4.5 cc. instead of 5 cc.

⁴ Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329. Sumner, J. B., *ibid.*, 1918, xxxiv, 37.

tralize and Nesslerize to 10 cc. volume 1 cc. of the supernatant liquid. If the reagents are free from ammonia the blank will show no color other than that due to the Nessler solution.

In Table I are figures obtained by running parallel determinations by the new method and by the Marshall-Van Slyke-Cullen aeration method.⁵

TABLE I.
Urea and Ammonia Nitrogen in Gm. per Liter of Urine.

		New method.	Marshall-Van Slyke-Cullen method.
1	Normal.....	2.04	2.01
2	"	2.39	2.42
3	"	6.03	6.08
4	"	4.51	4.50
5	"	6.95	6.95
6	"	5.02	5.03
7	"	4.20	4.16
8	Diabetic.....	5.88	5.68
9	"	4.48	4.48
10	"	5.24	5.35
11	Nephritic.....	8.73	8.55
12	"	6.17	6.25
13	"	6.21	6.13
14	"	15.15	15.04
15	"	5.89	5.90
16	"	11.56	11.52
17	"	4.90	4.95

CONCLUSION.

A method has been devised for the estimation of urea in urine which is rapid, accurate, and simple.

We wish to express our thanks to Mr. Hubbard and Mr. Blau of the Clifton Springs Sanitarium for furnishing us with pathological urines.

⁵ The figures obtained by the aeration method have been corrected for the ammonia nitrogen evolved by the action of potassium carbonate on the urease tablets during aeration. Under the conditions of the experiment the amount was 0.04 mg.

LYSINE AS A HYDROLYTIC PRODUCT OF HORDEIN.

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Hordein is the alcohol-soluble protein of barley, *Hordeum vulgare*. Determinations of the hydrolytic products of this protein have been made by Osborne and Clapp¹ and by Kleinschmitt.² These investigators isolated the basic amino-acids by the direct method of Kossel and found no evidence that hordein contains lysine. The complexity of Kossel's method makes it extremely difficult to isolate lysine when this amino-acid is present in but small quantities in proteins. This difficulty is shown by the results obtained in the earlier analyses of gliadin, the alcohol-soluble protein of wheat, which failed to reveal the presence of lysine. Later work by Osborne and Leavenworth³ resulted in the isolation of about 0.15 per cent of lysine from gliadin. Further work by Osborne, Van Slyke, Leavenworth, and Vinograd⁴ resulted in the isolation of 0.64 per cent of lysine while the indirect method of Van Slyke indicated the presence of 1.21 per cent of lysine in gliadin. A determination of the free amino nitrogen of gliadin by the method of Van Slyke and Birchard⁵ verified the latter result. That gliadin contains lysine was also indicated by the observation⁶ that white rats grow at a slow rate for a long time when gliadin is the only source of protein in the diet, while proteins which do not contain lysine fail to promote growth.

Nutrition experiments described by Osborne and Mendel⁶ show that hordein also promotes growth at a slow rate, which indicates

¹ Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907, xix, 117.

² Kleinschmitt, A., *Z. physiol. Chem.*, 1907-08, liv, 110.

³ Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1913, xiv, 481.

⁴ Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

⁵ Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913-14, xvi, 539.

⁶ Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.

that it contains lysine. The results of the present investigation corroborate this observation.

The hordein used in this investigation was prepared from barley flour by the method of Osborne.⁷ The protein was prepared by Mr. Gersdorff of this Laboratory. It was analyzed by the Van Slyke method and duplicate determinations indicated the presence of 0.77 and 1.01 per cent of lysine respectively. A free amino nitrogen determination made on hordein by the method of Van Slyke and Birchard⁸ also indicated the presence of 1.01 per cent of lysine.

The sulfur determination on the bases precipitated by phosphotungstic acid showed the presence of at least 1.18 per cent of cystine in hordein. This figure is undoubtedly too low since the cystine is partly decomposed during the hydrolysis of the protein. These results, as well as those previously reported, are given below. It is interesting to note how closely these figures agree with the latest results obtained on gliadin, which are also given.

Basic Amino-Acids in Hordein and Gliadin.

	Amino-acid from 100 gm. of protein.			
	Hordein.			Gliadin, ⁴ Van Slyke method.*
	Osborne and Clapp, ¹ Kossel method.	Kleinschmitt, ² Kossel method.	Present an- alysis, Van Slyke method.	
	gm.	gm.	gm.	
Cystine.....	Not isolated.	Not isolated.	1.18	1.17
Arginine.....	2.16	3.14	2.89	2.97
Histidine.....	1.28	0.51	2.14	2.19
Lysine.....	0.00	0.00	1.01	1.21

* Percentage of cystine estimated from cystine nitrogen obtained by Van Slyke method.

EXPERIMENTAL.

Analysis of Hordein by the Van Slyke Method.—Duplicate 3 gm. samples were used, each equivalent to 2.8311 gm. of moisture- and ash-free protein, and containing 16.92 per cent of nitrogen. Each sample, therefore, contained 0.4790 gm. of nitrogen. The

¹ Osborne, T. B., *J. Am. Chem. Soc.*, 1895, xvii, 539.

protein was dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solution for 24 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method. The results are given in Tables I and II.

TABLE I.

Analysis of Hordein, Van Slyke Method. Nitrogen Corrected for Solubility of Bases.

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.1156	0.1129	24.13	23.57	23.85
Humin N adsorbed by lime...	0.0041	0.0040	0.85	0.84	0.84
“ N in amyl alcohol extract.....	0.0011	0.0015	0.23	0.32	0.28
Cystine N.....	0.0039	0.0039	0.81	0.81	0.81
Arginine N.....	0.0250	0.0264	5.22	5.51	5.37
Histidine N.....	0.0184	0.0164	3.84	3.42	3.63
Lysine N.....	0.0042	0.0055	0.88	1.15	1.01
Amino N of filtrate.....	0.2355	0.2373	49.16	49.54	49.35
Non-amino N of filtrate.....	0.0703	0.0657	14.68	13.71	14.20
Total N regained.....	0.4781	0.4736	99.80	98.87	99.34

TABLE II.

Basic Amino-Acids in Hordein. Calculated from Van Slyke Analysis.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine.....	1.18	1.18	1.18
Arginine.....	2.74	2.89	2.82
Histidine.....	2.40	2.14	2.27
Lysine.....	0.77	1.01	0.89

Determination of the Free Amino Nitrogen in Hordein.—The solutions used for these determinations were prepared by dissolving 2 gm. of hordein by triturating with 25 cc. of 1 per cent acetic acid in a mortar. Diphenyl ether was used to prevent foaming and a correction made for the gas evolved by the reagents. The large deaminizing bulb of the Van Slyke apparatus was used and the nitrogen evolved measured in the micro-burette. The results are recorded in Table III.

TABLE III.

Free Amino Nitrogen of Hordein Compared with the Lysine Nitrogen.

Total N in sample analysed.	N gas in sample analysed.	Pressure.	Tempera- ture.	Amino N in sample analysed.	Ratio of amino N to total N.	Average ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	cc.	mm.	°C.	mg.	per cent	per cent	per cent
18.56	0.20	760	27	0.110	0.59	0.58	0.58
92.80	0.95	760	27	0.522	0.56		

SUMMARY.

1. The basic amino-acids of hordein have been determined by the method of Van Slyke.

2. The analyses indicate that hordein contains about 1 per cent of lysine which has hitherto not been shown to be present in this protein.

3. The free amino nitrogen in hordein has been determined and has been found to be equal to one-half of the lysine nitrogen as determined by the Van Slyke method.

4. The percentages of the different basic amino-acids in hordein are almost the same as those found in gliadin of wheat.

GAS TENSIONS IN THE TISSUES OF THE MOUTH.*

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(Received for publication, March 26, 1919.)

It is important in many problems to know the tensions of gases and volatile substances in the blood and tissues; *e.g.*, oxygen, CO₂, nitrogen ether, CO, etc. It occurred to us that a simple method of determining these tensions would be afforded by filling one of the body cavities or a subcutaneous connective tissue space with air or some other gas mixture. Diffusion must sooner or later establish gaseous equilibrium with the surrounding tissues; the composition of the gas in the cavity can then be determined by analysis.

The most easily available cavity is the mouth. By holding the cheeks blown out and breathing through the nose, which can be done for 15 or 20 minutes at a time, a closed cavity is obtained, for if the mouth were not shut off from the pharynx by the soft palate and tongue the cheeks would not remain distended. To fill the mouth with alveolar air it is necessary merely to make a deep expiration of which the last portion is retained in the mouth. To fill it with air of more than alveolar CO₂ tension, the breath is held for 20 or 30 seconds or longer, a deep expiration is made into a gas sampler,¹ and the cheeks are distended with the last part of the expiration. While this air is being held in the mouth, that in the sampler is analyzed. The air in the mouth is then drawn into the gas analyzer through a fine glass tube inserted between the lips. For initial concentrations of CO₂ higher than the body can

* The expenses of these investigations were defrayed in part by the Loomis Medical Research Fund.

¹ Henderson, Y., and Morris, W. H., *J. Biol. Chem.*, 1917, **xxi**, 221, Fig. 2.

produce the gas mixture is made in a bag and a sample analyzed before the mouth is filled.

The data which we obtained in this way regarding CO_2 are given in Table I.

From these observations it appears that the point of equilibrium for CO_2 in the mouth is about 7.5 per cent, or 54 mm. mercury. As saliva is rich in carbon dioxide it might perhaps tend to make this figure higher than that which would be correct for the tissues.

TABLE I.

Initial concentration of CO_2 .	Time held in mouth.	Final tension of CO_2 .
<i>per cent</i>	<i>min.</i>	<i>per cent</i>
Air.	11	2.64
"	11	2.65
5.7	5	6.3
6.9	10	7.3
7.65	4	7.65
7.65	8	7.4
7.8	10	7.55
10.8	4	10.3

In a few observations we have found the tension of CO_2 in saliva to be about 58 mm. of mercury and the content about 7 volumes per cent when determined without the addition of acid. The error from the CO_2 in the saliva is therefore probably inconsiderable.

Recent papers from this laboratory have shown that when the breath is held to the breaking point² or air is rebreathed³ until the CO_2 ceases to rise, a CO_2 tension of about 7.5 per cent is reached. It was pointed out that this is probably the CO_2 tension in the tissues, while that in the venous blood is about 6.6 per cent or 47.5 mm. For determining the oxygen equilibrium various mixtures of air and nitrogen were made. The data are given in Table II.

² Henderson, Y., and Prince, A. L., *J. Biol. Chem.*, 1917, xxxii, 325.

³ Laurens, H., *Am. J. Physiol.*, 1918, xlvi, 147.

TABLE II.

Initial concentration of oxygen.	Time held in mouth.	Final tension of oxygen.
<i>per cent</i>	<i>min.</i>	<i>per cent</i>
12.90	6	12.15
12.43	11	11.69
11.12	5	10.70
9.85	4	9.25
9.28	10	8.78
9.26	3	9.00
7.57	10	7.25
7.44	4	7.29
6.80	6	6.67
6.55	10	6.07
5.48	10	4.97
2.72	10	2.45
1.79	10	1.65
1.32	11	1.32
0.53	11	0.50
0.06	11	0.05

These figures seem to justify the conclusion that the point of oxygen equilibrium is not above, but possibly is considerably below 7 per cent or 50 mm. of mercury. The rate of diffusion of oxygen is much slower than that of CO₂ and the lower figures in Column 3 are therefore only approximate.⁴

⁴Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.

GAS TENSIONS OF THE ABDOMINAL CAVITY, WITH SOME EVIDENCE ON THE DIFFUSION OF GASES WITHIN THE BODY.*

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(Received for publication, March 26, 1919.)

The determination of the alveolar CO_2 in animals (dogs) is liable to considerable error, particularly in those conditions in which the gas tensions of the blood may be of especial significance. Still less can one judge of the oxygen supply to the tissues from analyses of alveolar air, or even of arterial blood.

We find that when air, or other gas mixture, is injected into the abdominal cavity, it comes after a time to have a tension of CO_2 equivalent to that of the arterial blood and alveolar air.

The diffusion of oxygen is much less rapid; owing to this fact and to the form of the oxyhemoglobin dissociation curve, the oxygen consumption by the peritoneum reduces the oxygen tension to 50 ± 5 mm.¹

The procedure was as follows: A definite amount of gas, usually 1 liter of air or nitrogen, was introduced by means of a hypodermic needle inserted through the abdominal wall in a lower quadrant. At intervals samples were withdrawn and analyzed by the apparatus previously described.² In no case has any injury to the peritoneum been apparent when the animal was later killed and autopsied. When blood samples were drawn for analysis, the femoral

* The greater part of this work was done under the War Gas Investigations of the Bureau of Mines and the Chemical Warfare Service, U. S. Army.

The expenses of these investigations were defrayed in part by the Loomis Medical Research Fund.

¹ Henderson, Y., and Stehle, R. L., *J. Biol. Chem.*, xxxviii, 67.

² Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 32.

artery on one side and the femoral vein on the other were exposed under local anesthesia. The venous blood was drawn by means of a hypodermic syringe without obstructing the blood flow in the vessel.

A general anesthetic was unnecessary and was not used, as the operation causes no discomfort. General anesthesia exerts so powerful an influence upon respiration and the blood gases as seriously to impair the validity of experiments on these functions. Pain, fear, or excitement likewise exert extremely disturbing effects and must be avoided absolutely. With care and gentleness, however, it is possible, and indeed easy, to carry out the exposure and opening of blood vessels and other operations under local anesthesia, without appreciable disturbance of either the physiological or psychological equilibrium of the animal.

Each of the following experiments is one of several in which nearly identical results were obtained. All the time intervals were noted from a clock which was set at 12 at the moment when the gas was injected into the abdomen. The alveolar air samples were taken by the Higgins-Plesch method by means of a mask and bag; the animal rebreathed 200 cc. of air for 30, 40, and 50 seconds. From these three analyses the two which agreed most nearly were averaged to obtain the percentages recorded in the protocols.

Every experiment here reported has been repeated at least once with results essentially identical with those of the protocol selected for publication.

Experiment 1.—Dog, female, 9 kilos.

Time.	Alveolar CO ₂ (mm. of mercury).	Incarcerated gases.	
		CO ₂ (mm. of mercury).	Oxygen (mm. of mercury).
11.25	38		
12.00			
1 liter of air introduced into abdominal cavity.			
12.30	35	24	
12.50	37	36	126
2.05	39	38	119
2.35	38	39	108
4.35	38	38	90

Experiment 2.—Dog, male, 16.5 kilos.

Time.	Alveolar CO ₂ (mm. of mercury).	Incarcerated gases.	
		CO ₂ (mm. of mercury).	Oxygen (mm. of mercury).
<i>Ars.</i>			
0			
1 liter of air introduced into abdominal cavity.			
7	38	38	66
30	37	39	53
48	36	34	52
54		37	56

Experiment 3.—Dog, female, 8 kilos.

Time.	Alveolar CO ₂ (mm. of mercury).	Incarcerated gases.	
		CO ₂ (mm. of mercury).	Oxygen (mm. of mercury).
11.55	41		
12.00			
1 liter of nitrogen introduced into abdominal cavity.			
12.10	39	13	10
12.20		19	14
12.30	40	31	20
12.40		39	26
12.50	42	39	31
1.00		40	39
1.10	39	38	42
1.20		38	44
1.30	41	40	45
2.30	40	39	46
3.30	41	40	45
4.30		38	46

Analyses to determine the oxygen content were made on three samples of this animal's blood. (The animal had an unusually small amount of hemoglobin, a fact without significance in this connection.)

	Oxygen vol. per cent
(a) Arterial blood (alveolar air O ₂ 95 mm, CO ₂ 40 mm.)....	12.2
(b) Blood equilibrated at 39°C. with room air + 5.55 per cent CO ₂ , i.e., O ₂ 145 mm. + CO ₂ 40 mm.....	12.6
(c) Blood equilibrated at 39°C. with air drawn from the abdomen, i.e., O ₂ 46 mm. + CO ₂ 40 mm.....	10.1
Difference between (a) and (c) (corresponding to a difference of oxygen tension of 95-46 = 49 mm.).....	2.1

Evidently a great fall of oxygen tension (49 mm.) is necessary before the blood gives up even a small amount (2.1 volumes per cent) of oxygen to the tissues. This fact, with the slow diffusion of oxygen and the consumption by the tissues, explains the low oxygen tension of the abdominal cavity. It probably approximates the local venous tension.

Experiment 4.—Dog, male, 16 kilos.

Time.	Alveolar CO ₂ (mm. of mercury).	Incarcerated gas CO ₂ (mm. of mercury).
11.45	37	
12.00		
1 liter of air introduced into abdominal cavity.		
12.30	36	6
1.00	38	8
1.30	37	10
2.00		11
3.00	39	17
4.00	38	22
5.00		28
6.00	37	32
7.00		35

The rate of diffusion between air and blood in this experiment was unusually slow. When this dog was killed and autopsied, a thick fat omentum was found covering the whole of the anterior visceral surface and preventing direct contact with the incarcerated air.

From these experiments it appears that when either air or nitrogen is used the equilibrium of CO₂ is reached within 1 hour. The tension of CO₂ in the abdominal air becomes the same as that of the alveolar air. The oxygen equilibrium is reached after an hour and a half when nitrogen is injected, but only after a much

longer period when air is used. Owing to the comparatively slow diffusion of oxygen through the tissues, the equilibrium tension of oxygen in the abdominal air is much below that of the alveolar air and arterial blood. It may approximate the local venous tension (cf. Experiment 3). The impediment offered by a large fat omentum is illustrated in Experiment 4; animals of this rare type, in which a constant tension of CO_2 is not attained in 1 hour, should be discarded.

Experiment 5 demonstrates that the CO_2 tension of the incarcerated air comes into equilibrium with that of the arterial blood.

Experiment 5.—Dog, male, 12 kilos. 1 liter of air was injected into the abdomen. 2 hours later analyses for CO_2 were made on the alveolar and abdominal airs. Tensions of 43 and 42 mm. respectively were found. Analyses for CO_2 were then made on the arterial and venous blood and on 3 samples of blood equilibrated at 39°C . to air containing (a) 22, (b) 40, and (c) 58 mm. of CO_2 . The analyses showed the CO_2 contents of these bloods to be in volumes per cent: arterial 38, venous 43, sample (a) 26, (b) 34, and (c) 44.

By plotting the CO_2 dissociation curve of the blood as defined by (a), (b), and (c), and noting on this curve the point corresponding to the CO_2 content of the arterial blood, a tension of 44 mm. is found, showing that the tension of CO_2 in the arterial blood was identical with that of the alveolar and abdominal air.

Experiment 6, on artificial acidosis, shows how closely changes of arterial CO_2 tension are followed by the abdominal air (cf. Columns 2 and 8 in the protocol).

Experiment 6.—Dog, male, 12 kilos. Procedure and analyses were the same as in Experiment 5. The blood Samples, (a), (b), and (c) were equilibrated at 37°C . to air containing 17, 40, and 60 mm. of CO_2 respectively, and analyzed; from these data the dissociation curves were plotted; the points in the curves corresponding to the CO_2 content of the arterial blood were then noted, and the tensions of CO_2 taken from the abscissæ of these points (Column 8).

Time.	Abdominal air tension of CO ₂ (mm. of mer- cury).	Blood CO ₂ analyses.					Indicated arterial CO ₂ tension (mm. of mer- cury).
		Ar- terial.	Venous.	Sample (a).	Sample (b).	Sample (c).	
		vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
12.00							
Injected 2 liters of air into abdomen.							
12.15	33						
1.35	41						
1.45	40	42	44	25	40	47	41
2.15							
Injected 10 cc. N HCl in- travenously, 2 cc. per min.							
2.45	22	24					
3.25	37						
3.45	37	37	45	25	38	44	37
5.05							
Injected 36 cc. N HCl in- travenously, 2 cc. per min.							
5.45	16	19	33	20	31	33	16
6.40	19	23	38	21	32	37	20

These data will be plotted in a figure to be published in a later paper on the CO₂ dissociation curves of the blood in experimental acidosis and some allied conditions. It is of interest to note in this experiment the correlation of the reduction of the CO₂ capacity of the blood and the lowering of the alveolar CO₂ tension; also the increasing difference between the CO₂ content of the arterial and venous blood, indicating a slowing of the circulation.

A criticism may be made of some of the data in Experiment 6 on the ground that, as found by some investigators,³⁻⁵ the CO₂ capacity of blood is influenced by variations in the oxygen tensions to which it is simultaneously exposed. In observations on

³ For older literature see Zuntz, N., in Hermann, L., *Handb. Physiol.*, Leipzig, 1882, iv, pt. 2, 81.

⁴ Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlvii, 244.

⁵ Parsons, T. R., *J. Physiol.*, 1917, li, 440.

this point, to be presented in a later paper, we have found that in whole blood the effect of pure nitrogen as compared with air on CO_2 capacity is barely appreciable. The effect on defibrinated blood,—which other investigators have used,—is much greater. Deoxygenation with hydrogen exerts a still greater influence upon the CO_2 capacity of the blood.

In repeated experiments it was found that when gas mixtures rich in CO_2 were injected into the abdomen the CO_2 was rapidly absorbed and eliminated through the lungs.

In several experiments also pure oxygen was injected into the abdomen, while the animal was rebreathing a small volume of air in a spirometer through a cartridge of alkali. Under these conditions CO_2 elimination from the lungs was not interfered with, while the peritoneum was the only avenue of entrance for oxygen into the body. In some experiments life continued under these conditions for several hours; the tension of oxygen in the re-breathed air fluctuated around 4 per cent. When the spirometer was filled with nitrogen, oxygen diffused outward from the lungs.

Experiment 7.—Dog, male, 12 kilos. 1 liter of nitrogen was injected into the abdomen. 5 hours later the animal was killed by a blow on the head. The abdominal gas was analyzed before death and at intervals for 18 hours thereafter.

	Incarcerated gases.	
	CO_2 (mm. of mercury).	Oxygen (mm. of mercury).
Before death.....	38	50
30 min. after death.....	49	47
1 hr. " "	56	45
18 hrs. " "	188	22

Experiment 7 shows that after death the consumption of any available oxygen, and the production, or liberation, of a much more than proportionate amount of CO_2 continue for at least 18 hours.

Mention may be made here of the fact that under etherization (without morphine) we have found the tension of ether vapor in the abdominal air (by the use of a Haldane gas analyzer, with a

pecially prepared paraffin oil as the absorbent) to be about 4 per cent or 29 mm. Boothby⁶ found a tension of 51 mm. necessary for full anesthesia in man, and thinks the figure 31 mm. which he calculates as that representing the estimate of Waller,⁷ and his coworkers to be too low. Our observation, however, is practically identical with that found by the latter investigators.

In experiments on carbon monoxide, in which the tension of CO in the inspired air was 0.1 per cent, distinct indications of this gas were found after 1 hour in the abdominal air.

After the foregoing observations were made we noticed Zuntz' article⁸ on the blood gases and found there that observations on our general topic have been made previously by Davy⁹ in 1823, by Leconte and Demarquay¹⁰ in 1859, and by others. (This topic was once of interest in relation to the ancient belief that exposure of a tissue to air was a cause of inflammation.) In general the data of these investigations, allowing for some obvious inaccuracies due doubtless to crude analytical methods, are substantiated by our findings.

CONCLUSIONS.

The contribution which we make consists in the demonstration that the CO₂ tension of the abdominal air soon becomes equivalent to that of the pulmonary alveolar air and of the arterial blood, and that under abnormal conditions (experimental acidosis) in which it is not easy or safe to cause the rebreathing necessary to obtain an alveolar sample, the CO₂ tension in the abdominal air falls in close correspondence to the arterial CO₂ tension.

Diffusion of oxygen through the tissues is much slower than is that of CO₂. The tension of oxygen in the abdominal air at equi-

⁶ Boothby, W. M., *J. Pharmacol. and Exp. Therap.*, 1913-14, v, 379.

⁷ Waller, A. D., Hewitt, F., Blumfeld, Gardner, J. A., and Buckmaster, G. A., *Anaesthetics*, Brit. Association for the Advancement of Science, London, 1911.

⁸ Zuntz, N., in Hermann, L., *Handb. Physiol.*, Leipsic, 1882, iv, pt. 2. 59.

⁹ Davy, J., *Phil. Tr.*, 1823, 496.

¹⁰ Leconte, C., and Demarquay, J., *Arch. gén. méd.*, 1859, xiv, 111, 424, 545.

librium is about 45 mm. of mercury, much below that of either the arterial or venous blood and corresponding therefore probably to that of the tissues.

When the air breathed contains carbon monoxide this gas appears in the abdominal air.

We find the tension of ether vapor in the abdominal air during anesthesia to be 29 mm. mercury.

A SYSTEM OF BLOOD ANALYSIS.

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(Received for publication, March 29, 1919.)

CONTENTS.

Introduction.....	81
Preparation of protein-free blood filtrates.....	82
Determination of non-protein nitrogen.....	87
" " urea.....	91
" " creatinine and creatine.....	98
" " uric acid.....	100
New Method for Determination of sugar.....	106

INTRODUCTION.

The main purpose of the research recorded in this paper has been to combine a number of different analytical procedures into a compact system of blood analysis, the starting point for which should be a protein-free blood filtrate suitable for the largest possible number of different determinations. It need scarcely be pointed out what a convenience and advantage it would be if one could take the whole of a given sample of blood and at once prepare from it a protein-free blood filtrate suitable for the determination of all or nearly all the water-soluble constituents, non-protein nitrogen, urea, creatinine, creatine, uric acid, and sugar.

In connection with our work on the problem we have also had in mind the desirability of reducing as far as practicable the amount of blood filtrate to be used for each determination, for by means of such reduction the total usefulness of the filtrate is correspondingly increased. There is no hard and fast limit as to the extent to which this reduction can be carried. It is doubtful, however, whether it is sound analytical practice regularly to use the smallest possible amount of material for each determination; whether, for example, blood filtrates corresponding to only 0.1 cc.

of blood should regularly be used for non-protein nitrogen determinations, because it may sometimes be advantageous or necessary to take no more. In this paper we deal chiefly with a semi microchemical scale of work representing only a moderate reduction of the quantities ordinarily taken for colorimetric work with the 60 mm. Duboscq colorimeter.

One of the main obstacles encountered in attempts to develop a definite system of blood analysis of the kind we have had in mind has been the determination of the uric acid: For several years we have had serious doubts as to the full trustworthiness of the uric acid results heretofore recorded in the literature; moreover, to be reasonably accurate the determination has required more blood (about 25 cc.) than can be obtained except in isolated special cases. A large share of the work involved in this research has therefore been a critical study of the uric acid determination; and a modification of the Folin-Denis-Benedict method has been developed which requires the filtrate from only 2 cc. of blood, and which we believe to be more dependable as well as more simple and convenient than the original method.

We have also satisfactorily solved the problem of how to make and keep standard uric acid solutions, and we have devised a new colorimetric method for the determination of sugar in blood.

The determinations included in this research, namely non-protein nitrogen, urea, creatinine, creatine, uric acid, and sugar, can all be determined in the filtrate obtained from 10 cc. of blood.

Preparation of Protein-Free Blood Filtrates.

The pivotal point in our projected general scheme of blood analysis was necessarily a searching review of the most promising methods which have been used for precipitating the blood proteins in connection with the various analytical procedures in common use. As a working principle or guide in this search we have first of all required that the procedure employed must permit the quantitative recovery of at least 10 mg. of uric acid and creatinine when added to 100 cc. of sheep, beef, or chicken blood, and that the total non-protein nitrogen must certainly be no higher than the figures obtained from a corresponding trichloroacetic acid filtrate representing a 10 per cent trichloroacetic acid concentration (in

the diluted unfiltered blood mixture)—or a corresponding 1.5 per cent *m*-phosphoric acid filtrate.

While we are not dependent on the urease method for the determination of the urea, we have, nevertheless, deemed it imperative that the blood filtrate must also be of such a character as readily to permit the use of the urease method for the determination of this important constituent. We do not claim to have exhausted this line of inquiry, for it is a laborious process to determine the merits and shortcomings of any particular reagent in connection with such a comprehensive program. None of the precipitation procedures described in recent years is free from serious shortcomings. Kahlbaum's phosphotungstic acid or sodium phosphotungstate (prepared by ourselves) met our requirements when used under certain very definite conditions, and for a time we concentrated our efforts on the standardization of these reagents and on the adaptation of the various analytical procedures to the blood filtrates obtained from them.

In connection with our work on sodium phosphotungstate we have discovered a new protein precipitant which probably has never before been used in blood analysis. We refer to it as new protein precipitant because so far as we have been able to learn it has never before been used in that capacity. This substance is tungstic acid. Tungstic acid, like sodium phosphotungstate or phosphotungstic acid, must be used in a definite way, but the necessary conditions are not difficult to find. Less than 1 gm. is used for the precipitation of the proteins from 10 cc. of blood, yet the precipitation is more complete than that produced by 10 gm. of trichloroacetic acid, and the filtrate obtained gives no trouble in connection with any of the determinatives so far investigated. Neither creatinine nor uric acid is carried down by the precipitate within the conditions to be described. As much as 20 mg. of uric acid may be added to 100 cc. of blood without incurring any loss by absorption.

The blood protein precipitation obtained by the help of tungstic acid is interesting. The precipitation is completed within a few seconds. When the mixture is shaken hard, the sound is almost like that of shaken mercury and the hardest kind of shaking will not produce more than a trace of foam. The precipitate is very fine, yet does not go through good filter paper and does not stop

up the pores. The filtration is slow, but the total amount of filtrate obtained is nearly as large as that obtained with trichloroacetic acid. If the precipitated mixture is heated in a water bath for 2 or 3 minutes, the precipitate settles spontaneously. With this modification, centrifuging can be substituted for the filtration as the supernatant liquid is water-clear and contains no more nitrogen than the unheated filtrate. For the present we do not care to recommend this process except for quantities of blood so small that one cannot afford to filter. The statement of Folin and Denis that no precipitation involving the use of heat is permissible is probably erroneous as has been pointed out by Bock, although it is true for the *m*-phosphoric acid precipitation and probably for many others. Unless some compelling advantage is gained by the use of heat, precipitation in the cold does seem to be the safer process.

There are many other points of interest to be investigated in connection with tungstic acid as a precipitant, but as these have no direct bearing on the problem of this research, further consideration of them here is omitted. As a precipitant for blood proteins we believe that tungstic acid will prove more useful than any other reagent yet proposed.

The precipitation of the blood proteins by means of our new reagent is made in the following manner. Transfer a measured amount of blood into a flask having a capacity of fifteen to twenty times that of the volume taken. Dilute the blood with 7 volumes of water and mix. With an appropriate pipette add 1 volume of 10 per cent solution of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) and mix. With another suitable pipette add to the contents in the flask (with shaking) 1 volume of $\frac{2}{3}$ normal sulfuric acid. Close the mouth of the flask with a rubber stopper and give a few vigorous shakes. If the conditions are right hardly a single air bubble will form as a result of the shaking. Much oxalate or citrate interferes with the coagulation and later with the uric acid determination. 20 mg. of potassium oxalate is ample for 10 cc. of blood. Citrate, except in the minimum amount, is to be avoided. When a blood is properly coagulated, the color of the coagulum gradually changes from pink to dark brown. If this change does not occur, the coagulation is incomplete, due, in every case we have encountered, to too much oxalate or citrate. In such an

emergency the sample may be saved by adding 2 normal sulfuric acid drop by drop, shaking vigorously after each addition and allowing the mixture to stand for a few minutes before adding more, until the coagulation is complete. Pour the mixture on a filter large enough to hold the entire contents of the flask and cover with a watch-glass. If the filtration is begun by pouring the first few cc. of the mixture down the double portion of the filter paper and withholding the remainder till the whole filter has been wet, the filtrates are almost invariably as clear as water from the first drop. If a filtrate is not perfectly clear, the first 2 or 3 cc. may have to be returned to the funnel. (Filter papers of the following diameters will meet all ordinary needs: 11, $12\frac{1}{2}$, 15, and $18\frac{1}{2}$ cm.)

It will be noted that the precipitation of the blood proteins is not made in volumetric flasks. Our procedure is adapted to the full use of practically all of a given sample of blood, for by this system 7, 9, or 12 cc. can be utilized just as well as 5 or 10. For this work we have devised a special blood pipette,¹ a sketch of which is given in Fig. 1. This is simply a 15 cc. pipette, graduated from the long tip into 1 cc. portions. The lower part is more or less like that of a volumetric pipette, thus permitting one to draw the blood directly from small, narrow bottles. We find it convenient to use three such pipettes; one for the blood, one for the sodium tungstate solution, and one for the sulfuric acid. The water used for diluting the blood may be measured with a cylinder.

The preparation of protein-free blood filtrates by this new process is so simple that no one need go astray, provided that the sodium tungstate and the $\frac{2}{3}$ normal sulfuric acid are correct. The only doubtful point is the quality of the sodium tungstate used. The acid is intended to set free the whole of tungstic acid with about 10 per cent excess (and to neutralize the carbonate usually present in commercial tungstates). A

¹ The pipettes are made for us by the Emil Greiner Co., New York.

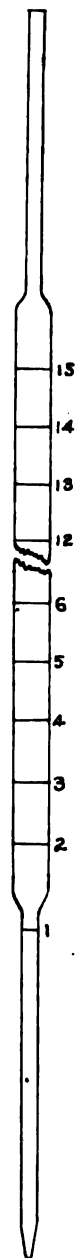


FIG. 1.

greater excess of sulfuric acid must not be used, for if this is the case a large part of the uric acid will be lost. A safe and convenient criterion is to test the blood filtrate obtained with Congo red paper. The reaction should be negative or at the most just perceptible. We have employed three different tungstates, and all worked equally well. The product we now use was obtained from the Primos Chemical Company, Primos, Pa.

The carbonate content of sodium tungstate is easily determined as follows: To 10 cc. of 10 per cent solution, add one drop of phenolphthalein and titrate with 0.1 normal hydrochloric acid. Each cc. of hydrochloric acid corresponds to 1.06 per cent of so-

TABLE I.

Comparison of Non-Protein Nitrogen in Blood Filtrates Obtained by Means of Trichloroacetic Acid and Tungstic Acid.

Source.	Mg. per 100 cc. blood.	
	Trichloroacetic acid.	Tungstic acid.
Human.....	35.7	31.5
"	32.4	28.2
"	35.4	33.0
"	42.0	42.0
"	50.7	48.6
Chicken.....	66.5	54.5
"	48.0	42.5
"	53.2	44.4
"	50.0	44.8
"	51.6	47.6

dium carbonate. The amount of acid required for the titration should not exceed 0.4 cc.

Our blood filtrates are nearly neutral, 10 cc. of filtrate requiring only about 0.2 cc. of 0.1 normal sodium hydroxide when titrated with phenolphthalein as indicator. If the filtrates are to be kept for any length of time, more than 2 or 3 days, they need some preservation. One or two drops of toluene or xylene is adequate for the filtrate obtained from 10 cc. of blood. Xylene seems to be fully as effective as toluene as a preservative.

The precipitation process just described works equally well with any kind of blood which we have yet tried—human, beef, sheep,

chicken, dog, and rabbit—and numerous comparisons with the trichloroacetic acid precipitation have shown that the non-protein nitrogen obtained by the process invariably tends to be lower than the figures given by trichloroacetic acid. The figures of Table I illustrate this point.

Determination of Non-protein Nitrogen.

The protein-free blood filtrates prepared by our new process lend themselves perfectly to nitrogen determinations by the direct Nesslerization process of Folin and Denis.² As a result of further experience with that method we are able to introduce certain modifications believed to represent improvements.

The acid digestion mixture is made as follows: Mix 300 cc. of phosphoric acid syrup (about 85 per cent H_3PO_4) with 100 cc. of concentrated sulfuric acid. Transfer to a tall cylinder, cover well to exclude the absorption of ammonia, and set aside for sedimentation of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part is clear and 50 to 100 cc. can be removed by means of a pipette. (It is not absolutely necessary that the calcium should be thus removed, but it is probably a little safer to have it done.) To 100 cc. of the clear acid add 10 cc. of 6 per cent copper sulfate solution and 100 cc. of water. 2 cc. of this solution are substantially equivalent to 1 cc. of the acid mixture previously described by Folin and Denis. We prefer this diluted acid, first, because the objectionable viscosity of the undiluted reagent is practically eliminated, and, second, because we now use for a nitrogen determination only 5 cc. (instead of 10 cc.) of blood filtrate, and 1 cc. of acid (corresponding to 0.5 cc. of the undiluted acid reagent).

The micro-Kjeldahl digestion is made as before in test-tubes. While we still have an abundant supply of Jena test-tubes we no longer use them for this digestion because the Pyrex ignition test-tubes are very much better in nearly every respect. Test-tubes having a capacity of about 75 cc. (200 × 25 mm.) are suitable for this purpose, and if made of Pyrex ignition glass are almost as good as those of pure silica. These test-tubes should be graduated

² Folin, O., and Denis, W., Nitrogen determination by direct Nesslerization, *J. Biol. Chem.*, 1916, xxvi, 473.

at 35 cc. and at 50 cc. on two sides, or by means of diamond marks going entirely around. The reason for this graduation is that we now Nesslerize in the digestion tube.

In micro-Kjeldahl digestions severe bumping is much more common than in ordinary macro-Kjeldahl digestions, but even in the latter the bumping phenomenon is often a source of serious difficulties. Glass beads, pumice stone, pieces of porcelain, etc., are used to remedy this trouble. For years ordinary quartz pebbles have been used in this laboratory, but at times these too have failed to prevent loss of a determination through sudden violent bumping. Occasionally a pebble may be hurled out of a 200 mm. test-tube by one intensive explosion. At other times no trouble at all is encountered. Langstroth,³ who seems to have encountered very severe and persistent bumping, resorts to the device of holding the test-tube in as nearly a horizontal position as the contents in the tube will permit; but since it takes half an hour to boil off the liquid which ordinarily can be boiled off in less than 10 minutes, that remedy cannot be considered satisfactory. Langstroth seems to have concluded that such bumping is peculiar to blood filtrates obtained by means of *m*-phosphoric acid, but the phenomenon is quite general. Prolonged boiling of pure water in any glass vessel will lead to the most intense bumping. The most important cause of bumping is certainly the condition of the test-tube. A new test-tube does not cause bumping, but if the same one is used over and over again in one session, the bumping becomes progressively worse. The worst kind of a test-tube (or Kjeldahl flask) can be made as good as a new one by thoroughly drying it; also by rinsing with alcohol.

The reason why dry test-tubes cause less bumping and why dry pebbles tend to prevent bumping is manifestly the presence of very fine pores filled with air in the test-tube and in the pebbles. Until this air has been driven out by heat, localized formation of steam occurs and the boiling is smooth and even, but as these pores are gradually filled with the liquid the bumping begins. By keeping on hand a sufficiently large number of dry test-tubes so that no one need be used more than twice in one session the bumping phenomenon, in the presence of a fresh (that is a *dry*) quartz pebble or piece of granite, is almost entirely eliminated.

³ Langstroth, L., Notes on Folin's direct Nesslerization method for the determination of nitrogen, *J. Biol. Chem.*, 1918, xxxvi, 377.

The Nesslerization process has also been simplified. The preliminary neutralization of the acid has been eliminated. The Nessler solution which we now use for all Nesslerizations is made as follows: The stock solution of mercuric potassium iodide can be made just as previously described. Dissolve 150 gm. of potassium iodide in 100 cc. of warm water, add 200 gm. of mercuric iodide, stir until the latter is dissolved, and dilute to a volume of about 1 liter; filter, if necessary, and dilute to a final volume of 2 liters. It is advantageous to make a large volume of this solution for a second sediment may form which takes a long time to settle.

The mercuric iodide obtainable from dealers frequently contains insoluble impurities (probably mercuric sulfide and mercurous iodide) which make it difficult to obtain a clear solution by the addition of potassium iodide. In such cases it is advisable to let the dissolved double iodide stand for 1 or 2 days and then filter, before diluting to volume.

Because of the difficulties encountered in obtaining high grade mercuric iodide, we have devised a new process for making the mercuric potassium iodide solution. This process is as follows: Transfer 150 gm. of potassium iodide and 110 gm. of iodine to a 500 cc. Florence flask; add 100 cc. of water and an excess of metallic mercury, 140 to 150 gm. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This whole operation usually does not take more than 15 minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters. If the cooling is begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water, and the finished solution can at once be used for Nesslerizations.

The cost of the chemicals called for in this process of making Nessler's solution is less than when starting with mercuric iodide, and the disagreeable impurities present in many samples of mercuric iodide are avoided.

From the stock solution of mercuric potassium iodide, made according to either of the processes described above, we prepare the final Nessler solution as follows: From completely saturated caustic soda solution containing about 55 gm. of NaOH per 100 cc. decant the clear supernatant liquid and dilute to a concentration of 10 per cent. (It is worth while to determine by titration that a 10 per cent solution has been obtained within an error of not over 5 per cent.) Introduce into a large bottle 3,500 cc. of 10 per cent sodium hydroxide solution, add 750 cc. of the double iodide solution and 750 cc. of distilled water, giving 5 liters of Nessler's solution.

The Nessler solution so obtained contains enough alkali in 15 cc. to neutralize 1 cc. of the diluted phosphoric-sulfuric acid mixture and to give a suitable degree of alkalinity for the development of the color given by ammonia at a volume of 50 cc.

(In other Nesslerizations, as in urine analysis when there is no acid to be neutralized, 10 cc. of the Nessler reagent per 100 cc. of Nesslerized ammonia solution is the correct amount.)

Concise Description of Non-Protein Nitrogen Determination.—Introduce 5 cc. of the protein-free blood filtrate into a dry 75 cc. test-tube graduated at 35 cc. and at 50 cc. Add 1 cc. of the sulfuric-phosphoric acid mixture described on page 87. Add a dry quartz pebble and boil vigorously over a microburner until the characteristic dense acid fumes begin to fill the test-tube. This is usually accomplished in from 3 to 7 minutes. When the fumes are unmistakable, cut down the size of the flame so that the contents of the tube are just visibly boiling, and close the mouth of the test-tube with a watch-glass or a very small Erlenmeyer flask. Continue the heating very gently for 2 minutes from the time the fumes began to be unmistakable, even if the solution has become clear and colorless at the end of 20 to 40 seconds. If the oxidations are not visibly finished at the end of 2 minutes the heating must be continued until the solution is nearly colorless. Such cases are very rare; the oxidation is almost invariably finished within the 1st minute. Allow the contents to cool for 70 to 90 seconds and then add 15 to 25 cc. of water. Cool further, approximately to room temperature, and add water to the 35 cc. mark. Add, preferably with a pipette, 15 cc. of the Nessler solution described above. Insert a clean rubber stopper and mix. If the solution

is turbid, centrifuge a portion before making the color comparison with the standard. The standard most commonly required is 0.3 mg. of N (in the form of ammonium sulfate) in a 100 cc. flask. Add to it 2 cc. of the sulfuric-phosphoric acid mixture, about 50 cc. of water, and 30 cc. of Nessler solution. Fill to the mark and mix. The unknown and the standard should be Nesslerized at approximately the same time. If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 30 gives the non-protein nitrogen in mg. per 100 cc. of blood.

Determination of Urea.

Investigations on the most satisfactory method for the determination of urea have been pursued for the last 2 or 3 years (partly with the assistance of G. L. Foster and Guy Youngburg). Much of the work done on the subject has been an endeavor to find a direct Nesslerization process without the use of Merck's blood charcoal. Our attempts have not resulted in any thoroughly satisfactory method because very small amounts of ammonia cannot be Nesslerized in the presence of either amino-acids or peptones. Direct Nesslerization, even with the help of charcoal, cannot be made except at the expenditure of more blood filtrate than is actually used in the final stages of the determination, and a strictly economical use of the blood filtrate we have considered a fundamentally important point in our system of blood analysis. Direct Nesslerization has therefore been abandoned in connection with the determination of urea in blood. Extensive use has also been made of the permutit extraction after first decomposing the urea with urease, but this process has proved somewhat fallacious with bloods in which the total urea nitrogen is small, as in many normal bloods, so this process also has been abandoned. Since probably no other determination will be as useful and important to the clinician as the determination of the blood urea, we have considered it of the utmost importance to get a method which is as simple as possible, but above all reliable. In this connection we have had in mind not only the needs of well equipped hospital laboratories, but also the needs of private practitioners.

For the hydrolysis of the urea we make use of jack bean urease, or the autoclave; for the isolation of the ammonia produced we employ aeration or distillation; thus we have four combinations any one of which will give satisfactory results. The autoclave process is, of course, not advantageous for single urea determinations, but on the other hand is distinctly useful when it is a question of a large series of determinations, or when creatine determinations are also to be made, because the hydrolysis of the urea can then be accomplished simultaneously with the conversion of the creatine into creatinine. The chief merit of the autoclave process for decomposing urea in blood filtrates lies perhaps in the fact that by its help one is sure to get all the urea nitrogen; the values obtained may be too high, but not too low. Yet the results obtained by the autoclave process are as a matter of fact usually identical and rarely as much as 1 mg. per 100 cc. of blood higher than those obtained by the urea process.

Urease Decomposition.—For the decomposition of urea by means of urease we use exclusively jack bean powder extracts and not so called purified or concentrated urease preparation. It is doubtless possible to prepare such, but those obtainable in the market are usually less active than an equal weight of jack bean powder, and of course are much more expensive.* An excellent urease solution can be prepared from jack bean powder in the following manner: Transfer to a 200 cc. flask or bottle about 3 gm. of permutit powder. Wash this by decantation, once with 2 per cent acetic acid, then twice with water. Add to the moist permutit in the flask 100 cc. of 30 per cent alcohol (35 cc. of 95 per cent alcohol mixed with 70 cc. of water). Then introduce 5 gm. of jack bean meal and shake for 10 minutes. Filter and collect the filtrate in three or four different small clean bottles. Set one aside for immediate use; it will remain serviceable at least 1 week at ordinary room temperature, if not exposed to direct sunlight. Put the others on ice where they will remain good for 3 to 5 weeks. The filtrate contains substantially the whole of the urease present in the jack bean powder and is very active. In the presence of a suitable phosphate mixture, 1 cc. added to 300 mg. of urea nitrogen at a volume of 200 cc. will yield 37 to 42 mg. of urea nitrogen

* The Arlington Chemical Co. supplies jack bean meal in a finer state of division than one can readily make by hand.

in 1 hour at 20°C. In 18 hours all the urea will be decomposed. The use of permutit makes the extract free from ammonia (5 cc. containing less than 0.01 mg.), nor does more ammonia develop on standing.

Urease decompositions of urea are never dependable except in the presence of some buffer mixtures by which the reaction of the solution can be kept within certain limits. The action of such mixtures is twofold. They not only accelerate the decomposition of the urea, but also prolong greatly the acting period of the enzyme. When urease solutions prepared as described above are added to urea dissolved in distilled water, it not infrequently happens that the enzyme acts for only a few minutes and then stops altogether, so that no more ammonia is obtained after 24 hours than after 15 minutes. That the enzyme is only dormant and not entirely destroyed is shown by the fact that on adding phosphate mixture to the solution after 24 or even 48 hours standing, renewed urea decomposition begins and then continues for a long time. The *Auzourease* found by Jacobi¹ to be present in blood serum represents probably nothing more or less than a preserving action of amphoteric serum proteins on the urease, action similar to that of phosphates.

In the course of our investigations on the determination of urea in blood filtrates by means of urease, it was accidentally found that other phosphates than those investigated by Van Slyke are equally good or better. When the titratable acidity of *m*-phosphoric acid blood filtrates was neutralized with sodium bicarbonate, the urease action on (added) urea was surprisingly active and long sustained. (The urea content of such blood filtrates can be determined conveniently both by the urease and by the autoclave processes.) In consequence of this discovery, a series of experiments was made with pyro- and *m*-phosphates, and our observations have led to the conclusion that a solution containing 140 gm. of sodium pyrophosphate (U.S.P.) and 20 gm. of glacial phosphoric acid per liter is probably better than any of the phosphate mixtures investigated by Van Slyke. We are at a loss for an explanation, for Van Slyke's mixtures cover the field sufficiently well from the standpoint of hydrogen and hydroxyl ion concentrations.

¹ Neumann, R., Über die Aktivierung der Soja-Urease durch menschliches Serum, *Biochem. Z.*, 1915, lxi, 134.

A thorough study of this subject has not been made, but it appears that the pyrophosphates are less injurious to urease than *o*-phosphates. One experiment may be cited.

Solutions containing mono- and disodium phosphate in the molecular ratios 1:1 and 1:2 were prepared. To 300 mg. of urea nitrogen in 200 cc. flasks were added (1) 5 cc. of phosphate 1:1, (2) 5 cc. of phosphate 1:2, and (3) 5 cc. of the pyrophosphate solution described above. To such mixtures were added water to 200 cc. and 1 cc. of urease solution (temperature 18°C.). Table II shows the results.

TABLE II.

Comparison of Effect of Different Buffer Mixtures on Rate of Hydrolysis of Urea by Action of Urease.

Buffer mixture.	Ammonia N.			
	15 min.	30 min.	1 hr.	19 hrs.
	mg.	mg.	mg.	mg.
Phosphate 1:1.....	5.7	9.6	21.4	266
" 1:2.....	5.7	9.5	19.8	180
Pyrophosphate.....	12.5	20.8	37.6	300

Determination of Urea by Urease Decomposition and Distillation.

Transfer 5 cc. of the tungstic acid blood filtrate to a *clean* and *dry* Pyrex ignition tube (capacity about 75 cc.). The graduated Pyrex tubes recommended for the non-protein nitrogen determination should never be used for urea determinations, because they have contained Nessler solutions and Nessler solutions leave behind films of mercury compounds which destroy the urease. If those tubes must be used, they should first be washed with nitric acid to remove the mercury films. Add to the blood filtrate two drops of the pyrophosphate solution described above or two drops of a molecular *o*-phosphate solution ($\frac{1}{3}$ molecular monosodium phosphate plus $\frac{2}{3}$ molecular disodium phosphate). Then add 0.5 to 1 cc. of the urease solution described on page 92 and immerse the test-tube in a beaker of warm water and leave it there for 5 minutes. The temperature of the water is not very important but should not exceed 55°C. The warm water can perhaps scarcely be said to be essential, for the hydrolysis is very rapid at room

temperature, but we nevertheless much prefer to use it. If no hot water is used, continue the digestion for 10 to 15 minutes, or as much longer as is convenient. The ammonia formed can be conveniently and quickly distilled into 2 cc. of 0.05 normal hydrochloric acid contained in a second test-tube. The second test-tube should not be so heavy as the ordinary test-tubes and should be graduated at 25 cc. A simple and compact arrangement for this distillation is indicated by Fig. 2. The test-tube which serves as a receiver is held in place by means of a rubber stopper in the side of which has been cut a fairly deep notch to permit the escape of air (and some steam). The rubber stopper serving as a holder for the receiver fits quite loosely to the delivery tube by means of which the two test-tubes are connected. The delivery tube must, of course, be so adjusted as to reach below the surface of the hydrochloric acid solution in the receiver before the distillation is begun.*

Add to the hydrolyzed blood filtrate a dry pebble, 2 cc. of saturated borax solution, and a drop or two of paraffin oil; insert firmly the rubber stopper carrying both delivery tube and receiver, and boil moderately fast over a microburner for 4 minutes. The size of the flame should never be cut down during the distillation, nor should the boiling be so brisk that the emission of steam from the receiving tube begins before the end of 3 minutes. At the end of 4 minutes slip off the receiver from the rubber stopper and put it in the position shown in Fig. 2. Continue the distillation for 1 more minute and rinse off the lower outside part of the delivery tube with a little water. Cool the distillate with running water, dilute to about 20 cc., and add 2.5 cc. of the Nessler solution described on page 90. Fill to the 25 cc. mark and compare in the colorimeter with a standard containing 0.3 mg. of N in a 100 cc. flask and Nesslerized with 10 cc. of the Nessler solution. The standard and unknown should always be Nesslerized as nearly simultaneously as practicable.

Calculation.—Multiply 20 (the height of the standard in mm.) by 15 and divide by the colorimetric reading to get the urea nitrogen per 100 cc. of blood. The reasons for this calculation are, of course, to be found in the fact that the standard containing 0.3

* The distillation apparatus can be obtained from Knott Apparatus Co., Boston.

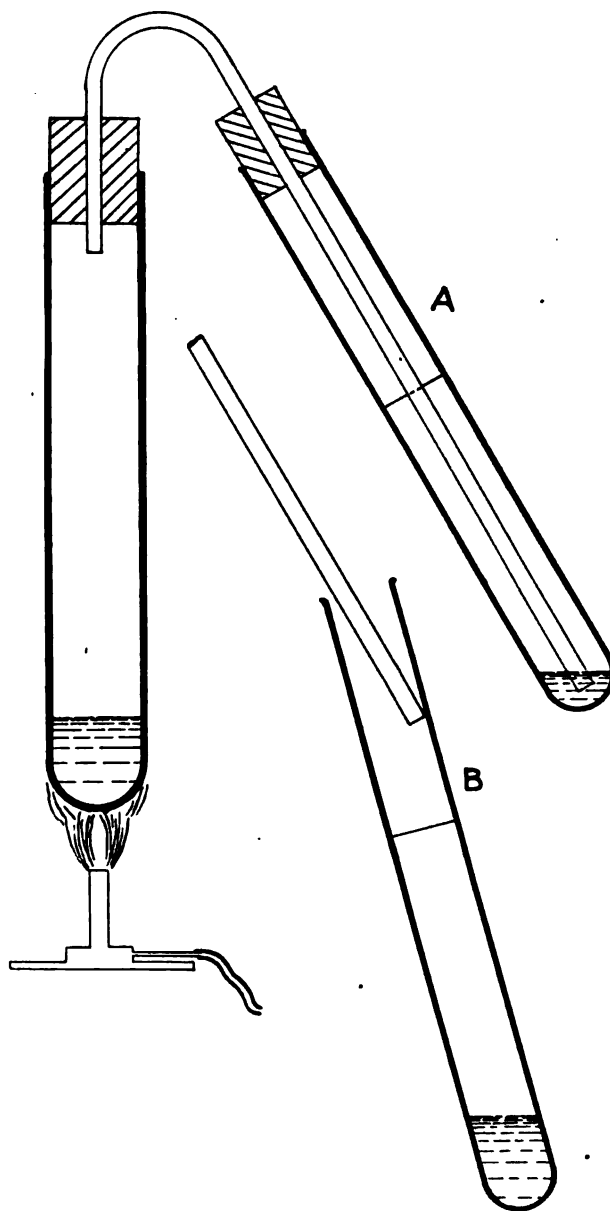


FIG. 2. A, at beginning; B, toward end of distillation.

mg. of N is diluted to 100 cc., while the unknown, which corresponds to 0.5 cc. of blood, is diluted to only 25 cc.

It is even more important in this distillation than in the non-protein nitrogen digestion that the Pyrex test-tube should not be in a condition that leads to bumping. Dry the tube, or rinse it with alcohol, after each determination.

Borax, the alkali used in this distillation, is strong enough to set free the ammonia, yet is so weak that the blank ammonia which it gives with 5 cc. of urease solution is scarcely any greater than that obtained by the aeration process.

It will be noted that no condenser is used in connection with the microdistillation described above. Since ammonia can be quantitatively recovered by means of an air current, it would seem that it should be recovered as easily by means of a current of steam, especially since the first part of the distillate, containing probably 90 per cent of the ammonia, is automatically condensed just as in ordinary macro-Kjeldahl distillations. A few experiments made along this line indicate that condensers are indeed superfluous even in macro-Kjeldahl distillations.

The other three modifications for the determination of urea in the blood filtrates can be referred to very briefly, for they will be used only by those who are already familiar with the principles and practices involved.

Urea Determination by Means of Urease and Aeration.—The decomposition of the urea is made in the same kind of a Pyrex test-tube and in the manner already described. 1 or 2 cc. of 10 per cent sodium hydroxide are added and the ammonia is aspirated into a test-tube graduated at 25 cc. and containing 2 cc. of 0.05 normal hydrochloric acid. The only precaution which experienced investigators are likely to overlook is that the rubber tubing used for connections needs to be rinsed with water before being used the first time, and, later also, if the tubing has been idle for any length of time. The talcum powder with which the inner and outer surface of rubber tubing is coated is probably the source of the trouble in the case of new rubber tubing. It is probably contaminated with ammonia.

Urea Determination by Means of Autoclave Decomposition.—To 5 cc. of blood filtrate in a 75 cc. test-tube is added 1 cc. of normal acid; the mouth of the test-tube is covered with tin-foil, and the

test-tube with contents is then heated in the autoclave at 150°C. for 10 minutes.

Allow the autoclave to cool to below 100°C. before opening. The ammonia is then distilled off exactly as in the first process described except that 2 cc. of 10 per cent sodium carbonate are substituted for the borax or it is removed by aeration in the usual manner. The autoclave process is of course only an adaptation of the process first recommended and then abandoned by Benedict for the determination of urea in urine. We are not prepared to say that in terms of per cent the results may not be as much too high in our blood filtrates as they were found to be in urine. An error of several per cent is, however, not at all important in the determination of the urea in blood. Whether one finds 15 instead of 14 mg. of urea nitrogen in human blood, or whether one obtains 3 instead of 2 mg. in 100 cc. of chicken blood is as yet of comparatively small consequence.

Determination of Creatinine and Creatine.


In this section we shall describe fairly obvious applications to our blood filtrate of Folin's colorimetric method for the determination of creatinine and creatine without thereby implying that the results so obtained are more accurate than the results which can be obtained by various other modifications which have been proposed during the past 2 or 3 years. The original methods as applied to blood were devised for the purpose of studying the absorption of creatinine and creatine and were adequate for that problem. Subsequent experience in many laboratories has shown that the method for the creatine gives results that are too high. The false step introduced in connection with the creatine determinations was undoubtedly the employment of picric acid as a protein precipitant, although at the time this seemed a peculiarly suitable process for securing the creatinine in concentrations then deemed necessary for reliable color comparisons. The process could perhaps be saved if it were worth while, for the cause of the high results is probably the formation of traces of hydrogen sulfide during the heating in the autoclave. The method is, however, now superfluous.

Determination of Preformed Creatinine.—Transfer 25 (or 50) cc. of a saturated solution of purified picric acid to a small, clean flask,

add 5 (or 10) cc. of 10 per cent sodium hydroxide, and mix. Transfer 10 cc. of blood filtrate to a small flask or to a test-tube, transfer 5 cc. of the standard creatinine solution described below to another flask, and dilute the standard to 20 cc. Then add 5 cc. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 cc. to the diluted creatinine solution. Let stand for 8 to 10 minutes and make the color comparison in the usual manner, never omitting first to ascertain that the two fields of the colorimeter are equal when both cups contain the standard creatinine picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate was added; it is therefore never advisable to work with more than three to five blood filtrates at a time.

When the amount of blood filtrate available for the creatinine determination is too small to permit repetition, it is of course advantageous or necessary to start with more than one standard. If a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution—using for such dilution a picrate solution first diluted with two volumes of water—so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxide.

One standard creatinine solution, suitable both for creatinine and for creatine determinations in blood, can be made as follows: Transfer to a liter flask 6 cc. of the standard creatinine solution used for urine analysis (which contains 6 mg. of creatinine); add 10 cc. of normal hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add four or five drops of toluene or xylene. 5 cc. of this solution contain 0.03 mg. of creatinine, and this amount plus 15 cc. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 cc. In the case of unusual bloods representing retention of creatinine, take 10 cc. of the standard plus 10 cc. of water, which covers the range of 2 to 4 mg. of creatinine per 100 cc. of blood; or 15 cc. of the standard plus 5 cc. of water by which 4 to 6 mg. can be estimated. By taking the full 20 cc. volume from the standard solution at least 8 mg. can be estimated; but when working with such blood it is well to consider whether



it may not be more advantageous to substitute 5 cc. of blood filtrate plus 5 cc. of water for the usual 10 cc. of blood filtrate.

Calculation.—The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown, in mm., gives the amount of creatinine, in mg. per 100 cc. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 cc. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate.

Determination of Creatine plus Creatinine.—Transfer 5 cc. of blood filtrate to a test-tube graduated at 25 cc. These test-tubes are also used for urea and for sugar determinations. Add 1 cc. of normal hydrochloric acid. Cover the mouth of the test-tube with tin-foil and heat in the autoclave to 130°C. for 20 minutes or, as for the urea hydrolysis, to 155°C. for 10 minutes. Cool. Add 5 cc. of the alkaline picrate solution and let stand for 8 to 10 minutes, then dilute to 25 cc. The standard solution required is 20 cc. of creatinine solution in a 50 cc. volumetric flask. Add 2 cc. of normal acid and 10 cc. of the alkaline picrate solution and after 10 minutes standing dilute to 50 cc. The preparation of the standard must of course have been made first so that it is ready for use when the unknown is ready for the color comparison. The height of the standard, usually 20 mm., divided by the reading of the unknown and multiplied by 6 gives the "total creatinine" in mg. per 100 cc. blood.

In the case of uremic bloods containing large amounts of creatinine 1, 2, or 3 cc. of blood filtrate, plus water enough to make approximately 5 cc., are substitutes for 5 cc. of the undiluted filtrate.

The normal value for "total creatinine" given by this method is about 6 mg. per 100 cc. of blood.

Determination of Uric Acid.

The colorimetric method for the determination of uric acid in blood, like the colorimetric method for the determination of creatinine in urine, has furnished a tangible starting point for much important research. With the introduction by Benedict of po-

tassium cyanide (or, as we prefer, sodium cyanide) for dissolving the silver urate, the uric acid method was materially simplified, and a new impetus was given to a widespread use of the process in researches of various kinds. For about 3 years doubts have been strong in this laboratory as to whether the method is really as reliable as it was at first believed to be, and in this laboratory at least we decided not to make further applications of it in research until these doubts could be removed. Our misgivings have proved in part unfounded and in part correct. Our fear that relatively large traces of uric acid are carried down with the blood proteins, during the coagulation process, have proved substantially groundless. On the other hand, it is certainly true that the precipitation of the uric acid from the concentrated blood filtrates by means of magnesia mixture and silver lactate (*i.e.*, essentially by Salkowski's process) is not quantitative, and the solubility of the silver urate is so large as to involve serious errors. On precipitating 0.1 mg. of uric acid from 10 cc. of solution an average loss of 50 per cent is encountered. By taking sufficiently large quantities of blood, 25 cc., the error due to the solubility of silver urate is of course largely eliminated, but the practical usefulness of the process is thereby much diminished. Another variable and uncontrollable source of error in the method is encountered during the concentration of the blood filtrates. If the total amount of water (acidified with acetic acid) to be boiled off does not exceed 100 cc. there is usually no destruction of uric acid, but when the volume is 200 to 400 cc. the losses, though variable, frequently amount to from 10 to 20 per cent, when starting with 0.1 or 0.2 mg. of uric acid. This source of error also can be eliminated almost wholly by taking aliquot portions of the blood filtrate (instead of the whole plus wash water). As a control method we have found the following process useful.

Heat about 160 cc. of water to boiling in a previously weighed beaker (capacity 500 cc.). Add 2 cc. of normal acetic acid, and add with pipettes 40 cc. of blood. Heat with constant stirring until the mixture is again boiling and continue the boiling for 2 minutes. Transfer beaker and contents to the scales, and add water until the total weight of the contents amounts to 200 gm. Mix and filter immediately. Transfer 100 cc. of the water-clear filtrate to an evaporating dish, add 1 cc. of 25 per cent acetic acid,

and boil down as rapidly as possible to a volume of about 5 cc. Transfer the residue to a 15 cc. centrifuge tube, rinsing with 1 to 2 cc. of 0.1 per cent lithium carbonate solution. Cool. Add 2 cc. of Benedict's ammoniacal silver magnesia mixture, stir for 2 minutes, and centrifuge. Decant as completely as possible the supernatant liquid. To the residue in the tube add 2 cc. of 5 per cent sodium cyanide solution; stir, add 10 to 13 cc. of water, stir, and centrifuge again. Transfer the supernatant liquid to a 100 cc. volumetric flask, and make the color comparison in the usual manner.

As a control method the process outlined above is good, and if we were dependent on the method for regular use we should cut it down and introduce corrections for the solubility of silver urate.

Before describing the determination of uric acid in tungstic acid blood filtrates we wish to describe the preparation of a new standard solution of uric acid—a solution the keeping quality of which we now, after 18 months of constant use, consider much superior to any other as yet devised. The solvent is 10 per cent sodium sulfite, and the keeping quality of the solution depends on the fact that the sulfite keeps the solution free from dissolved oxygen. The solution is prepared as follows:

Make 1 to 3 liters of a 20 per cent solution of sodium sulfite, let stand over night, and filter. Dissolve 1 gm. of uric acid in 125 to 150 cc. of 0.4 per cent lithium carbonate solution and dilute to a volume of 500 cc. Transfer 50 cc., corresponding to 100 mg. of uric acid, to each of a series of volumetric liter flasks. Add 200 to 300 cc. of water, then 500 cc. of filtered 20 per cent sodium sulfite solution, and finally make up to volume, and mix well. Fill a series of 200 cc. bottles, and stopper very tightly with rubber stoppers. The solution in a bottle which is opened daily will keep for at least 3 to 4 months. Our records kept for one larger bottle so used show that no measurable loss of uric acid had occurred at the end of 6 months. In unopened bottles we expect the uric acid to keep for many years.

The surplus 20 per cent sulfite solution should be diluted to concentration of 10 per cent and should then be transferred to another series of small, tightly stoppered bottles. This sulfite is added to the unknown in order to offset the sulfite content of the standard.

Solutions Required for Uric Acid Determinations.

1. The standard uric acid sulfite solution already described (3 cc. used for each series of determinations).

2. A 10 per cent sodium sulfite solution, also described (2 cc. used for each determination).

3. A 5 per cent sodium cyanide solution, to be added from a burette (2.5 to 5 cc. used for each series of determinations).

4. A 10 per cent solution of sodium chloride in 0.1 normal hydrochloric acid (10 to 20 cc. used for each series of determinations).

5. The uric acid reagent prepared according to Folin and Denis. A still stronger reagent is obtained by heating the sodium tungstate (100 gm.) and the phosphoric acid (80 cc.) plus water (700 cc.) for 24 hours, instead of 2 hours; but the advantage gained, about 20 per cent, is not needed. Dilute the solution to 1 liter.

6. A solution of 5 per cent silver lactate in 5 per cent lactic acid (4 to 5 cc. needed for each determination).

In our new method for the determination of uric acid the latter is precipitated directly from the filtrate, without any previous concentration. 20 cc. of filtrate corresponding to 2 cc. of blood are used. In describing the process we shall have to introduce a slight variation from the way we actually do it. This variation is due to the fact that we use a larger centrifuge than most laboratories possess and by means of which we are able to use 30 cc. test-tubes for the precipitation. Using the small 15 cc. centrifuge tubes, it is necessary either to precipitate 10 cc. of filtrate in each of two tubes or to make the precipitation in two 10 cc. installments.

To 10 cc. of blood filtrate in each of two centrifuge tubes add 2 cc. of a 5 per cent solution of silver lactate in 5 per cent lactic acid, and stir with a very fine glass rod. Centrifuge; add a drop of silver lactate to the supernatant solution, which should be almost perfectly clear and should not become turbid when the last drop of silver solution is added. Remove the supernatant liquid by decantation as completely as possible. Add to each tube 1 cc. of a solution of 10 per cent sodium chloride in 0.1 normal hydrochloric acid and stir thoroughly with the glass rod. Then add 5 to 6 cc. of water, stir again, and centrifuge once more. By this chloride treatment the uric acid is set free from the precipitate. Transfer the two supernatant liquids by decantation to a 25 cc. volu-

metric flask. Add 1 cc. of a 10 per cent solution of sodium sulfite, 0.5 cc. of a 5 per cent solution of sodium cyanide, and 3 cc. of a 20 per cent solution of sodium carbonate. Prepare simultaneously two standard uric acid solutions as follows:

Transfer to one 50 cc. volumetric flask 1 cc. and to another 50 cc. flask 2 cc. of the standard uric acid sulfite solution described above. To the first flask add also 1 cc. of 10 per cent sodium sulfite solution. Then add to each flask 4 cc. of the acidified sodium chloride solution, 1 cc. of the sodium cyanide solution, and 6 cc. of the sodium carbonate solution. Dilute with water to about 45 cc. When the two standard solutions and the unknown have been prepared as described they are ready for the addition of the uric acid reagent of Folin and Denis. Add 0.5 cc. of this reagent to the unknown and 1 cc. to each of the standards, and mix. Let stand for 10 minutes, fill to the mark with water, mix, and make the color comparison.

Calculation.—In connection with the calculation it is to be noted (a) that the blood filtrate taken corresponds to 2 cc. of blood, (b) that the standard is diluted to twice the volume of the unknown, and (c) that the standard used contains 0.1 or 0.2 mg. of uric acid. The blood filtrate from blood containing 2.5 mg. of uric acid will be just equal in color to the weaker standard. 20 times 2.5 divided by the reading of the unknown gives, therefore, the uric acid content of the blood when the weaker standard is set at 20 mm.

The two standards recommended were adopted on the basis of the experience gained from the analysis of more than 150 different samples of human blood. About one-third of these bloods was from soldiers and most of the others were obtained from the State Wassermann Laboratory through the courtesy of Dr. Hinton. The bloods unfortunately do not cover the wider range occurring among hospital patients. A moderate number of blood samples have been obtained from the Massachusetts General Hospital, and these reveal that the uric acid may sink to as low as 1 mg. of uric acid per 100 cc. of blood. It seems hardly worth while to prepare a third and weaker standard regularly in order to provide for such low uric acid values. A standard corresponding to the color obtained from 1.25 mg. of uric acid per 100 cc. of blood can be prepared within a couple of minutes as follows:

Transfer 1 cc. of 10 per cent sulfite solution, 3 cc. of 20 per cent sodium carbonate, 2 cc. of the acidified sodium chloride, 0.5 cc. of the sodium cyanide solution, and 25 cc. of the weaker one of the two regular standard solutions already on hand. Dilute to 50 cc. and mix. Or, simply add 5 cc. of 20 per cent sodium carbonate to 25 cc. of the regular weaker standard, and dilute to 50 cc.

If a low uric acid value is expected, an alternate procedure is to dilute the unknown to a final volume of 10 cc. with corresponding reduction in the amount of the reagents used.

Special attention should perhaps be called to one small yet essential variation in the process for developing the blue uric acid

TABLE III.

Comparison of Old and New Methods For Determination of Blood Uric Acid.

Source.	Mg. per 100 cc. blood.		
	New method.	Old method.*	
		Without solubility correction.	With solubility correction.
Human.....	2.6	2.2	2.4
"	3.5	3.4	3.6
Chicken.....	2.8	2.7	2.9
"	3.4	3.3	3.5
"	2.5	2.3	2.4
"	3.8	3.5	3.7

* Slightly modified.

color, a variation made necessary by the use of sodium sulfite. The uric acid reagent must invariably be added after, and not before, the addition of the sodium carbonate, because in acid solution the sulfite will itself give a blue color with the phosphotungstic acid.

It may also be worth while to mention that the peculiar increase in blue color obtained by the use of cyanide is not obtained in the presence of sulfite. Opinions will doubtless differ as to whether this is an advantage or disadvantage. The amount of color obtained from 2 cc. of blood is rather weak, and if we could conveniently have retained the intensifying effects of the cyanide we probably should have done so, though the fainter solutions can be

read just as readily and accurately as the stronger ones obtained by means of the cyanide. The antifading effects of the cyanide are retained.

New Method for Determination of Sugar.

It was originally our intention to incorporate some adaptation of Benedict's picrate method for the determination of sugar to our tungstic acid blood filtrates. But a few exploratory experiments showed that an intense and stable color reaction can be obtained by the application of the phenol reagent of Folin and Denis to cuprous oxide. The color obtained from a given quantity of sugar is far more intense than that obtained by the alkaline picrate reaction; so that a small fraction of a mg. of dextrose (1 or 2 cc. of blood filtrate) is all that is required for a determination of the blood sugar. Some difficulties were encountered in trying to find the conditions under which the extent of reduction is strictly proportionate to the quantities of sugar used; but, by a systematic study of the various factors involved, these difficulties were overcome and a rapid and convenient process was obtained.

The copper solution used for reduction is a weakly alkaline copper tartrate solution. Qualitatively this solution is an extremely sensitive reagent for traces of sugar, yet is not affected by creatinine or uric acid in quantities corresponding to 50 mg. of each per 100 cc. of blood. We are therefore inclined to regard our method as more accurate than any method as yet proposed for the determination of sugar in blood.

The picrate methods,⁷ whether we use Benedict's last modification or Myers' modification of Benedict's original method, in our hands give almost invariably results that are materially higher than the figures given by our new method. We are under the impression that the picrate methods are subject to sources of error similar to those encountered in Folin's original picrate method for blood creatine. The development of color in blood filtrates seems

⁷ Lewis, R. C., and Benedict, S. R., A Method for the estimation of sugar in small quantities of blood, *J. Biol. Chem.*, 1915, xx, 61. Myers, V. C., and Bailey, C. V., The Lewis and Benedict method for the estimation of blood sugar, with some observations obtained in disease, *ibid.*, 1916, xxiv, 147. Benedict, S. R., A modification of the Lewis-Benedict method for the determination of sugar in the blood, *ibid.*, 1918, xxxiv, 203.

not to proceed at the same rate of speed as the color derived from a corresponding amount of dextrose. If the heating is interrupted at the end of 2 to 3 minutes the value obtained for the blood sugar will be nearly 50 per cent higher than when the heating is continued for 10 minutes or more. Such quantitative variations are not encountered in our process when equal amounts of dextrose in the form of pure sugar and of blood filtrate are heated, except that the reduced copper is, of course, more extensively precipitated and visible in the pure sugar solution. It need scarcely be stated that added sugar is quantitatively recovered by our method.

Solutions Needed for Determination of Sugar in Blood.

1. *Standard Sugar Solution.*—Dissolve 1 gm. of pure anhydrous dextrose in water and dilute to a volume of 100 cc. Mix, add a few drops of xylene or toluene, and bottle. If pure dextrose is not available, a standard solution of invert sugar made from cane sugar is equally useful. Transfer exactly 1 gm. of cane sugar to a 100 cc. volumetric flask; add 20 cc. of normal hydrochloric acid and let the mixture stand over night at room temperature (or rotate the flask and contents continuously for 10 minutes in a water bath kept at 70°C.). Add 1.68 gm. of sodium bicarbonate and about 0.2 gm. of sodium acetate, to neutralize the hydrochloric acid. Shake a few minutes to remove most of the carbonic acid and fill to the 100 cc. mark with water. Then add 5 cc. more of water (1 gm. of cane sugar yields 1.05 gm. of invert sugar) and mix. Transfer to a bottle; add a few drops of xylene or toluene, shake well, and stopper tightly. The stock solution made in either way keeps indefinitely. Dilute 5 cc. to 500 cc., giving a solution 10 cc. of which contain 1 mg. of dextrose or invert sugar. Add some xylene. Use 2 cc. for each determination.

2. *Alkaline Copper Solution.*—Dissolve 40 gm. of anhydrous sodium carbonate in about 400 cc. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid and when the latter has dissolved add 4.5 gm. of crystallized copper sulfate; mix, and make up to a volume of 1 liter. If the carbonate used is impure, a sediment may be formed in the course of a week or so. If this happens, decant the clear solution into another bottle.

3. *Phosphotungstic-phosphomolybdic Acid*.—Transfer to a large flask 25 gm. of molybdenum trioxide (MoO_3) or 34 gm. of ammonium molybdate $(\text{NH}_4)_2(\text{MoO}_4)$; add 140 cc. of 10 per cent sodium hydroxide and about 150 cc. of water. Boil for 20 minutes to drive off the ammonia (molybdic acid sometimes contains large amounts of ammonia as impurity). Add to the solution 100 gm. of sodium tungstate, 50 cc. of 85 per cent phosphoric acid, and 100 cc. of concentrated hydrochloric acid. Dilute to a volume of 700 to 800 cc.; close the mouth of the flask with a funnel and watch-glass. Boil gently for not less than 4 hours, adding hot water from time to time to replace that lost during the boiling. Cool and dilute to 1 liter. This solution is identical with the phenol reagent of Folin and Denis. For use in connection with the determination of blood sugar dilute 1 volume (100 cc.) of the reagent with one-half volume (50 cc.) of water and one-half volume (50 cc.) of concentrated hydrochloric acid.

4. *Saturated Sodium Carbonate Solution*.

The determination of blood sugar is carried out as follows: Heat a beaker of water to vigorous boiling. Transfer 2 cc. of the tungstic acid blood filtrate to a test-tube (20 m. \times 200 mm.) graduated at 25 cc. The graduated test-tubes used as receivers when distilling off the ammonia in urea determinations (p. 95) are suitable for this work. Transfer 2 cc. of the dilute standard sugar solution to another similar test-tube. Add to each tube 2 cc. of the alkaline copper tartrate solution. Heat in the boiling water for 6 minutes. Remove the test-tube and add at once (without cooling), preferably from a graduated pipette, 1 cc. of the strongly acidified and diluted phenol reagent. This should be done as nearly simultaneously as possible; it is not advisable to use one standard for a set of more than four determinations. The purpose of the added hydrochloric acid in the reagent is to dissolve the cuprous oxide. Mix, cool, and add 5 cc. of saturated sodium carbonate solution. An intense blue color is gradually developed which will remain unaltered for several days. Dilute the contents of both test-tubes to the 25 cc. mark, and after at least 5 minutes make the color comparison in the usual manner.

The depth of the standard (in mm.) multiplied by 100 and divided by the reading of the unknown gives the sugar content, in mg., per 100 cc. of blood.

TABLE IV.

Sample Analyses of Protein-Free Blood Filtrates Obtained by Means of Tungstic Acid.

No.	Mg. per 100 cc. blood.					
	Total N.	Urea N.	Uric acid.	Preformed creatinine.	Total creatinine.	Sugar.
1	26	10	1.3	1.5	6.0	89
2	26	13	1.0	1.4	5.3	100
3	28	12	1.1	1.2	6.7	98
4	28	12	2.2	2.0	5.7	83
5	29	13	3.3	1.5	6.0	86
6	29	11	2.6	1.4	5.2	95
7	29	13	1.6	1.4	6.0	85
8	30	13	2.4	1.6	5.5	82
9	30	14	4.1	1.7	5.3	82
10	32	15	2.8	1.6	5.4	91
11	32	15	3.4	1.4	5.3	97
12	32	13	2.4	1.7	6.0	104
13	33	17	2.0	1.3	4.8	83
14	33	16	2.5	1.6	5.7	105
15	33	15	1.1	1.6	5.5	95
16	34	16	0.8	1.3	6.1	119
17	34	16	2.6	1.5	5.9	106
18	35	17	2.1	1.6	6.0	89
19	35	17	2.0	1.4	5.5	77
20	35	18	2.0	1.7	5.7	86
21	35	18	2.9	1.6	5.8	95
22	35	17	3.2	1.4	5.5	94
23	35	18	2.5	1.5	6.0	89
24	35	19	2.2	1.5	5.3	91
25	35	22	3.5	1.4	5.7	87
26	35	17	2.3	1.7	6.7	83
27	35	18	1.6	1.3	6.5	104
28	36	17	2.8	1.5	5.2	100
29	37	18	2.1	1.5	5.5	94
30	38	18	2.2	1.7	5.4	95
31	39	18	2.6	1.8	6.7	103
32	39	18	2.9	1.5	6.0	87
33	40	18	2.0	1.6	6.0	98
34	40	20	2.6	1.7	5.6	95
35	41	19	4.8	1.5	5.9	93
36	41	19	4.2	2.5	6.6	109
37	43	19	2.2	1.7	6.3	78
38	139	106	5.4	12.5	19.4	99
39	147	115	8.9	11.0	20.5	170
40	275	237	14.3	13.6	27.2	157

The copper solution is adjusted to give proportionate reductions with 0.12 to 0.4 mg. of dextrose. This covers the range of hypoglycemic and hyperglycemic bloods. But in extreme cases it is better to use 3 or 1 cc. of the filtrate, instead of 2 cc., adding water to the standard or to the unknown so as to equalize the concentration of the alkaline copper.

NOTE ON THE DETERMINATION OF UREA IN URINE BY DIRECT NESSLERIZATION.

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Merck's blood charcoal was a necessary reagent in determination of urea in urine by the direct Nesslerization process of Folin and Denis. By using a urease preparation sufficiently free from nitrogenous materials the urea nitrogen can be Nesslerized without any charcoal treatment. The process is as follows:

Wash about 3 gm. of permutit in a flask once with 2 per cent acetic acid, then twice with water; add 5 gm. of fine jack bean meal and 100 cc. of 30 per cent alcohol. Shake gently but continuously for 10 to 15 minutes and filter. The filtrate contains practically the whole of the urease and extremely little of other materials. Add 1 cc. of this urease solution to 1 cc. of diluted urine (dilution usually 1:10) in a test-tube and digest in a beaker of warm water (40–55°C.) for 5 minutes or at room temperature for 15 minutes. It is preferable, but not necessary, to add a drop of one of the phosphate solutions described in the preceding paper¹ to the mixed contents in the test-tube at the beginning of the digestion. The buffer mixture is particularly desirable if the digestion is to be made at room temperature. At the end of the digestion period transfer the contents of the test-tube to a 200 cc. volumetric flask, diluting to a volume of about 150 cc. Add 1 mg. of N in the form of ammonium sulfate to another 200 cc. flask; to this standard add 1 cc. of the urease solution and dilute to about 150 cc. Then add 20 cc. of the Nessler solution prepared according to the directions given in the preceding paper (p. 89). Dilute to volume and make the color comparison. The ammonia nitrogen is, of course, included in the figure obtained.

A few explanatory remarks may be added. Many kinds of biological nitrogenous materials, particularly amino-acids, pep-

¹ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

tones, and albumins, prevent the development of the color reaction given by ammonia and Nessler's reagent. This was first discovered in attempts to determine by direct Nesslerization the ammonia formed in pancreatic digestion mixtures. If very little such nitrogenous material is present, the result obtained is deceptive for then the color is merely diminished and the error will not be detected. The careful observer will find, however, that in such cases the color obtained tends to be visibly more greenish and less distinctly red than the standard. Because of the serious interference caused by albuminous materials it may be thought that the procedure described above is not applicable to albuminous urines, but by a series of determinations we have convinced ourselves that even urines very rich in albumin have in fact so little in comparison with the amount of urea present that correct results are invariably obtained by direct Nesslerization.

Because of the extremely low nitrogen content of our urease preparation, it is not really essential that the urease should also be added to the standard ammonia solution, but we have thought it best to recommend that it be added simply as a precaution against the possible occurrence of less good urease preparations. In recommending the addition of the urease to the standard as well as to the urine we have also had in mind the probability that many will omit the use of the permutit when making the alcoholic urease extracts and will then have variable small traces of ammonia in their extracts.

The reason why the urease decomposition is better made in test-tubes rather than in volumetric flasks is to avoid failure due to the use of flasks which have been used for Nesslerization purposes. Such flasks may look perfectly clean, but, unless they are rinsed with nitric acid, they will contain enough mercury compounds to destroy entirely the urease and scarcely a trace of ammonia is obtained.

The method described above was worked out about 2 years ago and has been used with our medical students for two seasons. The publication has been delayed because it was our intention to incorporate it in a larger research. After consultation with Dr. W. S. McEllory who has independently devised a method very similar to ours, it has seemed better to publish the method without further delay.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

VI. OBSERVATIONS ON THE FAULTS OF CERTAIN DIETS COMPARABLE TO THOSE EMPLOYED BY MAN IN PELLAGROUS DISTRICTS.

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PLATE 1.

(Received for publication, February 24, 1919.)

In making an analysis of the relation of the diet to pellagra, the results of which have been presented in the present series of papers, we began by showing the nature of the dietary faults of a mixture of two seeds, one a cereal (maize) and the other a legume seed (navy bean). It was shown that with this mixture of two natural foodstuffs, which are both recognized as wholesome foods, the same kinds of deficiencies exist as we had previously determined for each of the more important seeds employed as foodstuffs when fed as the sole source of nutrients; *viz.*, a decided shortage of the mineral elements, calcium, sodium, and chlorine; a relative shortage of the dietary factor fat-soluble A; and a protein content of rather low biological value as compared with such foods as milk, meats, and eggs. The detailed evidence in support of these deductions was presented in the first paper of the series (1). The first limiting factor in determining the nutritive value of a mixture of maize and navy beans is the mineral content, and the remaining two factors which are unsatisfactory are each below the optimum to about the same degree. All three of these factors must be supplemented before satisfactory nutrition can be secured.

We had previously made the observation that fairly good nutrition, as shown by capacity to grow to the full adult size and produce and rear young, could be secured with monotonous diets derived from mixtures of the leaf of the plant with the seed (2), whereas with mixtures of seeds only little or no growth could be

secured in young animals (3), and adult animals fail to maintain their vitality when restricted to monotonous diets of this character (4). In the fourth paper of the series the dietary properties of mixtures of seeds derived from three to five sources were made clear. It was demonstrated that there is almost no supplementary action between the ordinary seeds which are employed as human foods with respect to the mutual improvement of their inorganic content, and that they do not supplement each other with respect to fat-soluble A (3).

That the proteins of the seeds which are of importance in nutrition are of low biological value for the support of growth was made clear by more elaborate experiments in the third paper of the series (5). In the second paper (6) it was demonstrated that physiological well being is not maintained when animals are limited to an intake of any one or more dietary factors, allowing but a small margin over the actual daily requirements for the repair of the endogenous waste. In a recent communication it has likewise been made clear that the supplementary relationship between the proteins of the seeds generally is much lower than one might logically expect, so that the protein mixture derived from a diet containing seeds from a number of different plants is in general of low value for transformation into body proteins. There have been observed however, a few mixtures of two seeds in certain proportions, in which the quality of the proteins is nearly if not quite the equivalent of milk proteins in value (7).

The solution of the problems discussed in the foregoing papers forms a basis for deductions of far reaching importance in the relation of the diet to pellagra. Our data all support the view that with the exception of a single dietary complex (fat-soluble A) each whole seed which is of importance in the nutrition of man in America contains sufficient of any one or more chemically unidentified dietary factors to supply the needs of the growing young rat. According to the working hypothesis adopted by McCollum and Davis (8), and adhered to as the simplest one by means of which our experimental data with the rat could be interpreted, there are but two such complexes; *viz.*, the substance which is protective against xerophthalmia (fat-soluble A), and that which is protective against beri-beri (water-soluble B).

Our experimental studies have now progressed so far as to enable us to assert with confidence that a satisfactory diet cannot be secured from mixtures containing any number of seeds or products derived from the milling of seeds together with tubers, edible roots, and meats (Chart 4, Lot 2147). The vegetable foods which may be classed as seeds, tubers, and roots are all functionally storage organs, and their content of active protoplasm is relatively small in comparison with their bulk because of the large amount of reserve food material laid down in them. They may be sharply contrasted with the leaf of the plant, which except in special cases is not a repository for reserve proteins, carbohydrates, and fats, but represents, aside from its skeletal tissues, functionally active protoplasm (9). The leaf has very different dietary properties from those possessed by the tissues which are modified as storage organs, and in many instances at least represents complete foods for those types of animals whose digestive tracts are so capacious as to permit them to eat a sufficient amount of bulky material. As stated above, we have been able to prepare fairly satisfactory diets for an omnivorous animal, the rat, from these two types of vegetable foods together, *i.e.*, leaves and seeds, but never from the group of vegetable foods which are functionally storage organs (10).

From this experience we have been led to differentiate sharply between two classes of foods which are usually collectively designated as vegetables. Leaves are constituted so as to correct the dietary deficiencies of the storage tissues, whereas the seeds, tubers, and roots fail to supplement mutually each other's deficiencies with respect to either the inorganic moiety or the fat-soluble A. They do in some degree mutually enhance the quality of each other's proteins, but to a lesser degree than we had supposed before the completion of a large amount of experimental work directed toward the quantitative comparison of the protein mixtures derived from pairs of seeds in considerable number.

Mixtures of seeds, or of seeds, tubers, and roots, will in all cases require supplementing with respect to calcium, sodium, and chlorine among the inorganic elements, and fat-soluble A (compare Charts 3 and 4 with Chart 5). In most such mixtures the quality of the proteins will likewise be sufficiently poor to require improvement before the optimum well being can be secured.

We are now in possession of a considerable amount of knowledge concerning the distribution of the dietary factor, fat-soluble A, in animal tissues. The body fats of the ruminants probably always will be found to be richer in this substance than the body fats of the omnivora because of the greater intake of it in the food (11). Muscle tissue has been found to be very poor in fat-soluble A (4, 12) but the fats from the glandular organs, *i.e.*, intracellular fats, are a good source of it. It follows, therefore, that muscle tissue such as round steak should not supplement mixtures of vegetable foods which belong to the storage organ group with respect to fat-soluble A, and in our experience this proves to be the case. The inorganic content of muscle tissue resembles in a general way that of the storage organs of plants except in its very low content of magnesium. It is too poor in calcium, and to a lesser degree in sodium and chlorine, to support the optimum well being in an animal. Muscle tissue fails to supplement the seeds, tubers, and roots on the inorganic side (Chart 3). The protein content of muscle tissue is high and the proteins are probably of high biological value, and, except as respects palatability, it is only in improving the quality of the protein content of the ration that the addition of meats of this class enhances the value of a mixture of products derived from the storage organ group of plant products.

These considerations indicate the basis for our distinction between two groups of foodstuffs. One of these, which includes milk, eggs, and the leafy vegetables, we have designated as "protective foods," in order to call attention to their special importance in the diet (10). They are protective in that they are so constituted with respect to their inorganic content, content of fat-soluble A, and the quality of their proteins that they correct in great measure when used in sufficient amounts the faults of the remainder of the food mixture irrespective of the extent to which it is derived from either seed, tuber, or root products. We have been able to plan satisfactory diets of naturally occurring foods only by the inclusion of one or more of these protective foods. The other group of natural foodstuffs includes all seeds and seed products, such as the cereal grains and their milling products (wheat flour, corn-meal, polished rice, etc.), the legume seeds, tubers, edible roots, nuts, fruits, and such cuts of meats as come from muscle tissue.

In all cases where we have attempted to correct the dietary deficiencies of a seed mixture by the addition of leaf only we have not secured results so good as with milk, especially with such amounts of leaf as would be acceptable in the human diet (compare Charts 5 and 8). The leafy foods are eaten by Europeans and Americans only in a very water rich condition, and it is difficult to secure the consumption of enough to correct the deficiencies in the remainder of the diet. With animals, when we have fed dry powdered mixtures containing as much as 25 to 40 per cent of the diet derived from leaf and the remainder from plant products of the storage organ class, the nutrition has been very good in some instances, but not all combinations will be equally valuable (4). Eggs are decidedly poorer in calcium than are the leaves or milk, when only the part exclusive of the shell is considered. The shell serves as a source of lime to the developing chick. Eggs do not, therefore, supplement food mixtures derived from storage tissues with respect to calcium to the degree that milk and leafy vegetables do.

Even in such types of diet as contain one or more of the protective foods in fairly liberal amounts, it is certain that for such rapidly growing species of animals as the hog and rat the inorganic content is not entirely satisfactory, although it may be good enough to enable the animal to perform all the functions of growth and reproduction in a way which, in the absence of definite knowledge of what the species is capable of, we should regard as normal. We have been accustomed to regard as normal an achievement in vigor and well being both in man and animals which falls far short of that seen in exceptional cases. The most important inorganic deficiency in seed, tuber, and meat mixtures is calcium, and this is so pronounced that we are of the opinion that even in those human dietaries in which such calcium-rich food as milk are used in fair liberality, the intake of calcium may be still below the optimum, and that a direct addition of this element in the form of the carbonate or lactate might be of distinct benefit in human nutrition except perhaps in those regions where the water is unusually rich in calcium salts. Since civilized man usually adds sodium chloride to his foods to suit the taste, the shortage of sodium and chlorine in the diet of man presents no problem. An addition of calcium could be most conveniently made to our

foods through the use of a mixture of equal parts of common salt and of calcium carbonate in the kitchen and on the table.

A question which has never been answered to the satisfaction of physiologists is: How much protein should the diet contain in order to maintain physiological well being? At about the time when the question was being most discussed, the chemistry of the proteins was developed to a point which made it clear that there were great differences in the biological values of the proteins from different sources, depending on their yields of certain amino-acids. This makes futile any attempt to establish a particular intake of protein which may represent the minimum, optimum, or maximum amount consistent with maintenance of "normal" vitality and health. The quality of the proteins must be known before anything can be said about the amount of protein necessary. From biological tests we now know that the proteins of the pea or navy bean are worth only about half as much for growth in the rat as are equal amounts of proteins from one of the cereal grains, and that the latter have about half the value for the conversion into body proteins which can be shown for the proteins of milk. The relative values of the proteins from different sources, as well as the absolute values of certain of them, are just now becoming appreciated.

There are two opposing views regarding the amount of protein which will produce the best results. Those who advocate the low protein diet point to the "specific dynamic action" of protein, through which it stimulates metabolism. They believe that a high consumption of protein furnishes pabulum for the development of an excessive growth of putrefactive bacteria, with the result that toxic or irritating products of the degradation of certain amino-acids are absorbed in amounts sufficient to cause damage to the tissues (13). It has been recommended that man should, in adult life, take only such an amount of protein as will cover the endogenous loss due to tissue metabolism, together with a not well defined "margin of safety" (13). The opponents of this view regard a liberal protein allowance as essential to vigor and aggressiveness, and point to the use of liberal amounts of meat by the peoples who have been characterized by greatest achievement (14). *Among all the progressive peoples of the world the food supply is derived to a greater or less extent from dairy products,*

and this portion rather than the meat eaten we have come to regard as of peculiar importance in improving the quality of the diet. In order to test this question we conducted a series of experiments, employing rats which were about 9 months old, or about one-fourth through the normal span of life for this species, and were in excellent nutritive condition (5). They were fed diets which were fairly satisfactory in all respects except that the protein content was not far from the actual amount required for the maintenance of body weight for a few weeks. We observed unmistakable signs that the vitality of the animals was rapidly lowered on such a dietary régime. This was shown especially by the rapid aging and short span of life. Even though the initial body weight was approximately maintained for a period of 3 months or more, distinct signs of aging were always apparent within 5 to 10 months. 3 months in the life of a rat correspond to about 8.4 per cent of the average span of life. It can be readily appreciated that if harmful effects in corresponding degree follow the adherence by man to such low protein diets they would not become apparent within the time covered by any experiment yet conducted upon a diet squad, few of which have been restricted to any experimental diet beyond 6 months. A reputed satisfactory outcome of such experiments cannot be accepted as evidence that men on diets which furnish but a small margin of protein over the actual maintenance requirements are so nourished as best to promote health. Aging at two to four times the rate observed in the most satisfactorily nourished would escape observation in any experiment on man with which we are familiar.

The results of experiments with grown men restricted to experimental diets for a few weeks or months do not form a safe basis for drawing conclusions as to the quality of the foods employed. Certain conclusions may be warranted from general observations on children living on faulty diets, and important deductions may safely be drawn from the experiences of large groups of people living upon more or less restricted lists of foodstuffs. Beyond this we must be guided in human nutrition by the results of animal experimentation, in which the conditions can be made sufficiently rigid to bring into stronger contrast the faults of certain types of diets as contrasted with others. It is certain that the injurious effects of certain dietary practices are very real and yet not

promptly apparent. The debilitating effects of faulty diets may vary in their severity from such as will produce polyneuritis or xerophthalmia or scurvy within a few weeks, at one extreme, to such as will cause nervousness and restlessness in varying degree, susceptibility to disease, and the acquisition of all those characters such as roughness of the skin, thinness and coarseness of hair, and attenuation of form which accompany the process of aging at a distinctly greater rate than would be the case were the diet of a highly satisfactory character.

We have much evidence that in case there is a close approximation of the actual physiological minimum for any factor during growth, such as one or more of the essential inorganic elements or one of the unidentified dietary essentials, lack of ability to meet the more strenuous demands of reproduction and the suckling of young will be observed, and the tendency will be great for the individual to be carried off suddenly either by disease, or, as frequently happens, by causes which are not readily determinable (compare Charts 8 and 9).

All our experience with diets of low protein content have indicated that animals do not remain in a state of optimum well being even when the content of protein is sufficiently high to maintain in certain individuals the initial body weight over as much as 10 per cent of the normal span of life. We believe that health and vigor are promoted by a liberal intake of protein of good quality better than by any diet in which there is a tendency towards parsimony with respect to this dietary factor. It should not be lost sight of, however, that there are other factors in nutrition which are of equal importance with protein, and that if the optimum well being is to be attained the diet must be rightly constituted with respect to all its parts. In addition to this the prompt elimination of the fecal residues is essential and is a great relief to the tissues of the entire body.

With an understanding such as we now have of the nature of the faults of diets of different types, and an appreciation of the fundamental importance of deriving the constituents of the diet from the right sources, this being of much greater importance than composition as revealed by chemical analysis, one is in a position to interpret the relation of pellagra to diet.

Goldberger (15) has emphasized the fact that the diet of those living in districts in which pellagra is common is lacking in sufficient amounts of certain foodstuffs, especially milk, eggs, meats, and the legume seeds. In many instances bolted wheat flour, degerminated corn-meal, polished rice, sugar, syrups or molasses, sweet potatoes, and meat, principally pork, form almost the entire list of foods eaten by families during the winter season, at the end of which new attacks of pellagra are regularly seen. From what has been said it will be evident that the diet of the pellagrous is deficient in four respects, and that the nature of these is well understood. They are the deficiencies of the plant products which belong to the storage organ group, but more pronounced because of the prominent place which milling products, which represent the endosperm of the seed, find in such diets. Products such as bolted flour, degerminated corn-meal, and polished rice are decidedly poorer in inorganic elements than are the seeds from which they are derived; their proteins appear to be of poorer quality than are those of the cell-rich structures near the periphery, or of the germ, and they are almost devoid of fat-soluble A and very poor in water-soluble B. Whereas diets derived from whole seeds, tubers, and edible roots contain sufficient phosphorus to meet the requirements of the most rapidly growing species of animal, such as the rat, and the limiting inorganic elements are calcium, sodium, and chlorine, it may be that in diets in which the degerminated and decorticated cereal products are employed in liberal amounts, and where in addition starch, sugar, and molasses are regularly used freely, phosphorus or iron or both may likewise become important deficiencies (16).

Goldberger attempted to solve the problem of whether pellagra is due to lack of something essential in the typical "pellagrous" diet by a direct experiment on man (15). He restricted men to a diet prepared from bolted wheat flour, degerminated corn-meal, polished rice, starch, sugar, syrup, pork fat, sweet potatoes, cabbage, collards, turnip greens, and coffee, and at the end of 5½ months five of the eleven men who took this diet were diagnosed as exhibiting incipient signs of pellagra. That the disease was actually produced has been emphatically denied by McNeal (17).

In another experiment Goldberger and fifteen of his associates made heroic attempts to infect themselves with material from the

lesions of pellagra, and with excreta from pellagrins, but without success. The experimenters were, however, taking a diet of good quality while these attempts were being made (18).

Still more convincing evidence that the diet is at least an important predisposing factor in the etiology of pellagra is furnished by the experience of Goldberger in improving the diets in institutions in which the disease was common. These diets were observed to consist largely of degerminated seed products, tubers or roots, and fat pork, together with minimal amounts of leafy vegetables, fruits, eggs, meats, and milk, and the legume seeds. On modifying the diets of orphanages and of an insane asylum by the addition of lean meat, milk, eggs, and peas or beans, the condition with respect to pellagra steadily improved, and the disease promptly disappeared. New cases were admitted from without and the sick were mingled with the well, but after the improvement of the diet no new cases developed (15).

Those who have had extensive experience with pellagra are in agreement in the matter of the fundamental importance of dietary treatment together with any other method of management of pellagrins, and the assertion has been made by Roussel that without dietary measures all remedies fail (19). The results obtained by Goldberger point clearly to the belief that the disease develops because of some one or more faults in the diet. They afford no basis, however, for judging as to the nature of these faults, whether they are in the nature of a lack of a sufficient amount of one or more chemically unidentified dietary essentials of a specific character, as is known to be the case with beri-beri and the xerophthalmia of dietary origin, or whether pellagra may be the result of taking a diet faulty in respect to the quality or quantity of protein, relative shortage of one or more of the essential inorganic elements, or of the recognized unidentified dietary essentials as contributing factors.

In his earlier papers Goldberger expressed the view that: "On the whole, however, the trend of available evidence strongly suggests that pellagra will prove to be a 'deficiency' disease very closely related to beri-beri" (15). Chittenden and Underhill (20) reported the production in dogs of a condition suggestive of pellagra in man by restricting the animals for periods of from 2 to 8 months to a diet of crackers, peas, and cottonseed oil. They

formulated the conclusion that: "From the facts enumerated the conclusion seems tenable that the abnormal state may be referred to a deficiency of some essential dietary constituent or constituents, presumably belonging to the group of hitherto unrecognized but essential components of an adequate diet."

In the fifth paper of this series we reported the results of a study of the nature of the dietary faults of a mixture of bolted wheat flour, peas, and cottonseed oil, and found that it was an incomplete food, but that it was rendered complete for the support of normal growth in the young rat by the addition of purified protein, certain inorganic salts (NaCl and CaCO_3) and fat-soluble A (in butter fat). It is of course not satisfactorily established that the condition produced in dogs by the diet of Chittenden and Underhill was actually the counterpart of pellagra in man, strikingly similar as the results appear. McCollum, Simmonds, and Parsons (21) hold the view that if the condition produced in the dogs of these investigators is actually to be regarded as experimental pellagra, it cannot be regarded as caused by the lack of an unidentified dietary essential, since the only one of these necessary for completing the diet (for the rat) is that contained in butter fat, and the latter substance is not curative for any condition resembling pellagra, but for a specific eye disease, xerophthalmia (6).

In his most recent studies Goldberger and his associates examined the diets of pellagrous and non-pellagrous families in villages in South Carolina, and found that the diet of the non-pellagrous contained more milk, fresh meats, eggs, butter, and cheese than did the diets of pellagrous families, and that the calorific value of the diets of the former households was somewhat higher than of the latter (22). Animal proteins were eaten more liberally and cereal proteins were eaten less abundantly by the non-pellagrous than by the pellagrous households. The pellagrous households had a distinctly smaller supply of fat-soluble A, and a somewhat smaller supply of water-soluble B than did the non-pellagrous, and the inorganic content of the diets of the latter were of less satisfactory character than those of the former households. *We do not regard a moderate shortage of one or another of the chemically unidentified dietary factors as of greater gravity than faulty character in any other dietary factor.* Our studies of the several foodstuffs lead us to agree with Goldberger's interpretation of the quality of the diets of pellagrous and non-pellagrous households in all respects.

From the observations which we have made concerning the chemical factors which the diet must contain in order to be adequate for the support of growth in the young, or the maintenance of physiological well being in the adult, together with the results of our studies of the qualities of each of the more important kinds of natural foodstuffs, we are not able to account for the etiology of pellagra on the assumption that it is a disease which is due to the lack of a specific substance or substances of unknown chemical nature, as are without question beri-beri and xerophthalmia (6). This follows from the fact that, with the exceptions of certain manufactured food products which are derived from the endosperm of the decorticated grains, *any natural foodstuffs of the class of seeds, tubers, edible roots, or leafy parts of the plant, are so constituted that they can be supplemented by means of three kinds of purified food additions of known nature; viz., protein, certain salts, and fat-soluble A, so as to be complete for the nutrition of the young rat throughout the growing period.* This has been demonstrated to be true not only for each of the ordinary human foods but likewise for such mixtures as form the monotonous diets of the pellagrous (see Charts 1, 2, 3, and 4).

It is necessary, therefore, that we choose between two alternatives in arriving at an opinion concerning the etiology of pellagra. We have the assurances of Goldberger and his associates (15) that a diet such as that described in Charts 1 to 3, and having the qualities described in the preceding paragraph, has produced incipient pellagra experimentally in man, but this claim has been disputed by other competent observers (17). In our experimental work with the diet of peas, crackers (wheat flour and fat), and cottonseed oil, which in the experience of Chittenden and Underhill produced in dogs a condition resembling pellagra in man, produced in rats only general malnutrition, without the skin changes, diarrhea, or pathological changes in the mucosa of the alimentary tract. Are we to accept the view that pellagra is actually produced by a deficiency of something necessary to the normal nutrition of man but not necessary for the rat? The possibility that the dogs of Chittenden and Underhill were infected is not excluded, and an infectious agent may well have established itself in animals restricted to a diet so faulty as one derived from crackers (wheat flour and fat), peas, and cottonseed oil. Goldberger seems to have

safeguarded his experimental men against infection, and it is unfortunate that a sufficient number of undisputed authorities were not called into consultation to forestall the possibility of a question arising concerning the accuracy of the diagnosis of pellagra, such as McNeal has raised (17).

We are left in the situation which has arisen in the discussion of the etiology of scurvy. It has been clearly shown that there is no difficulty in repeating the experimental work which demonstrated that a guinea pig will develop severe scurvy (or some syndrome resembling it) on a number of diets on which the rat will thrive during the growing period. Does this mean that the guinea pig requires one or more chemical complexes for its nutrition that are dispensable to the rat? There is no doubt that the guinea pig normally takes a diet rich in succulent vegetables, and which produces bulky, easily eliminable feces. The rat and swine, as well as man, thrive on certain diets which leave little indigestible residue. Such special requirements in the guinea pig make it next to impossible to compare this species with man or the rat in similar dietary studies. The experimental data obtained with the guinea pig must be used with caution in reasoning concerning the etiology of human scurvy.

In our attempts to produce in animals a condition analogous to pellagra in man we have not been successful, but have observed only a generalized poor condition instead. The evidence is practically nil that Chittenden and Underhill's dogs suffered all the pathological changes which they record solely as the result of chemical faults in the diet. Our experiments with their diet shows it to be incapable of maintaining satisfactory nutrition because of faults in the dietary factors. The possibility of an infection in their animals is not excluded, and is indeed rendered probable if we grant that lowered vitality predisposes to infection. These reasons together with the lack of positive proof that the men restricted in Goldberger's experiments to a diet similar to that described in Chart 1 were actually developing pellagra, warrant, we believe, our accepting as probably correct the conclusions of the Thompson-McFadden Commission (23) and of Jobling and Peterson (24) that pellagra is caused by an infectious agent, and that unless it has been introduced into a district there may develop such a condition of lowered vitality from faulty diet or other de-

bilitating influence as would predispose one to an attack, without the appearance of the disease. The debilitating effects on animals of diets derived from cereals, tubers, roots, and any food products formed from the milling of grains together with legume seeds and meats, are so striking that we believe similar diets would produce in man a susceptibility to infectious diseases such as tuberculosis or pellagra. We have come to hold the view, as the result of our studies of diets of the type common in pellagrous households, that the predisposing influence for both is in general the same, and the character of the unsanitary conditions surrounding the individual may determine which of these two diseases he will develop.

From the studies which we have described in this series of papers we are enabled to point out definitely the relative values of several foods as correctives in the diet of the pellagrous. Hitherto the legume seeds and lean meat were classed with milk and eggs in this respect, and nothing was said about the unique qualities of the leafy vegetables as supplements for food mixtures derived from plant products of the storage organ group. It is clear that the most important food to be recommended for consumption in pellagrous districts is milk, because of its cheapness as compared with the same protective value in foods from other sources and its threefold corrective character as contrasted with meat which enhances the type of diet found in the pellagrous household only with respect to the protein factor, and eggs which are not so good as milk because of their lower calcium content. The legume seeds, notwithstanding their high content of protein, are without any appreciable value for improving the diets which predispose to pellagra, because of the poor quality of their protein and their failure to supplement a diet derived from vegetable foods of the storage tissue class in other respects.

Both meats and eggs are more expensive sources of protection against faulty diet than milk. An effective campaign of education should be conducted in all districts where diets of a character likely to predispose to pellagra are common, informing the people about the great benefits to health from regular and very liberal use of leafy vegetables. This would be a movement toward the establishment of dietary practices resembling those of the more nearly vegetarian groups of Chinese and Japanese, and if in addition the inclusion of a suitable amount of milk in the diet can be secured,

not only would pellagra disappear, but the general health of the people would be promoted.

The prevalence of pellagra in certain parts of the South rather than in other sections of the country is probably closely connected with the development of the modern milling industry. This places in the grocery store the degerminated and decorticated part of the grain. The rise of the sugar industry offers for human consumption both sugar and molasses in quantities unheard of until recent years.

The widespread practice of growing a cash crop (cotton), and of depending on the retail store for the greater part of the food supply

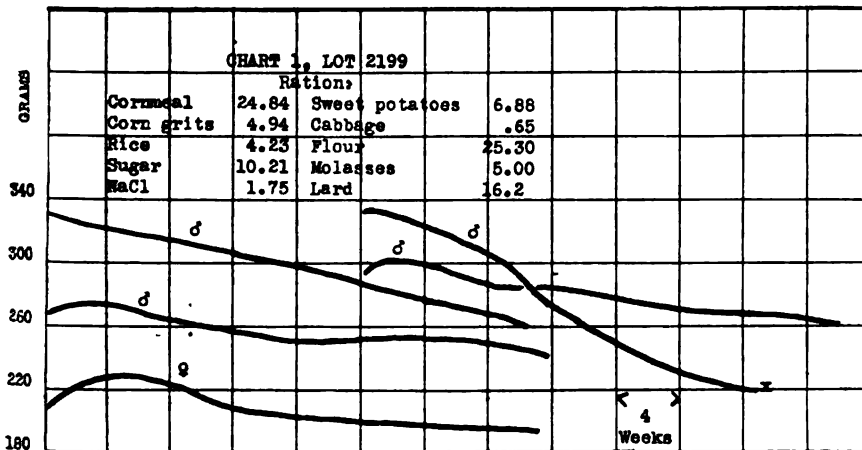


CHART 1.

rather than of engaging in diversified farming appears to be in great measure responsible for the existence of pellagra. The food products which can be handled commercially without hazard are not in general satisfactory foodstuffs unless properly supplemented with certain others which correct their deficiencies.

Chart 1.—Lot 2199 illustrates the failure of adult rats to maintain normal nutrition on a diet closely similar to that employed by Goldberger in his attempt to produce experimental pellagra in man. The only difference was that he employed cabbage, turnip top greens, and collards, whereas we replaced the two latter leafy vegetables by cabbage, but the total dry matter derived from a leaf source was the same in both diets.

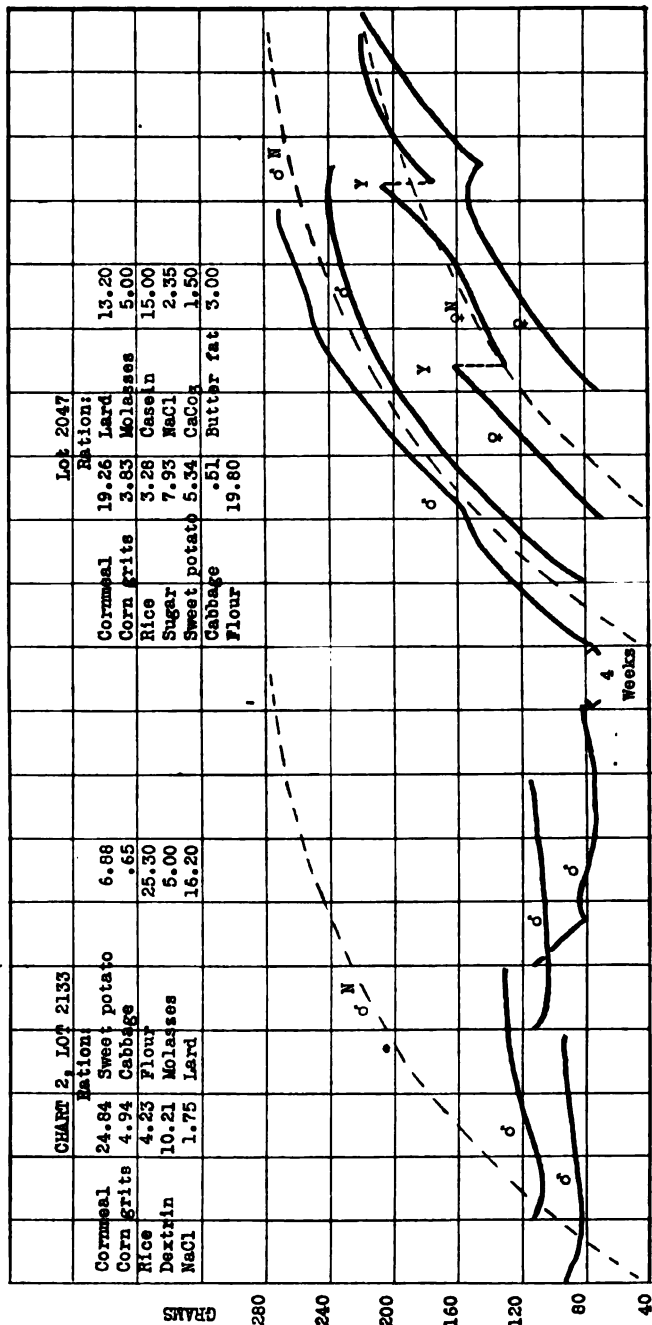


CHART 2.

Judging from such studies as we have made it appears that all leaves have much the same dietary properties, except such as contain obnoxious or poisonous products.

This diet afforded wide variety, palatability, and as far as chemical analysis could show, a satisfactory composition in every respect, but it proved unsatisfactory because it was derived too largely from the group of plant products which are functionally storage tissues. Less than 3 per cent of the dry matter of the diet was derived from leaf.

At the end of the feeding period these animals looked very old and rough haired, but were not emaciated. They were then about 16 months old, or somewhat less than one-half through the normal span of life, which is about 36 months. The diet, therefore, failed to maintain for long the characteristics of youth. The animals did not show any signs of a "deficiency" disease as beri-beri or xerophthalmia, for no specific lesion could be detected. There was nothing suggestive of the lesions seen in pellagra. The skin appeared healthy, and there was no diarrhea or abnormal appearance of the mucosa. It is certain that no combination of products derived from the endosperm of seeds, together with tubers and edible roots, will form a satisfactory food mixture for the support of growth in the young or of satisfactory nutrition in the adult. It seems reasonable to suppose that animals restricted to this type of diet would be rendered susceptible to infections of low order of virulence, against which well nourished individuals would be immune.

The cabbage, beets, turnips, and potatoes employed were dried at about 70°C. and ground. The potatoes were thoroughly steamed before drying.

Chart 2.—Lot 2133 shows the failure of young rats to grow on a diet which was similar in composition to that employed by Goldberger in his efforts to produce pellagra experimentally in man (15), except that cabbage was the only leaf product, and dextrinized starch replaced cane sugar. It consisted of corn-meal grits, rice, dextrin, bolted wheat flour, molasses, sweet potato, cabbage, baking powder, and lard.

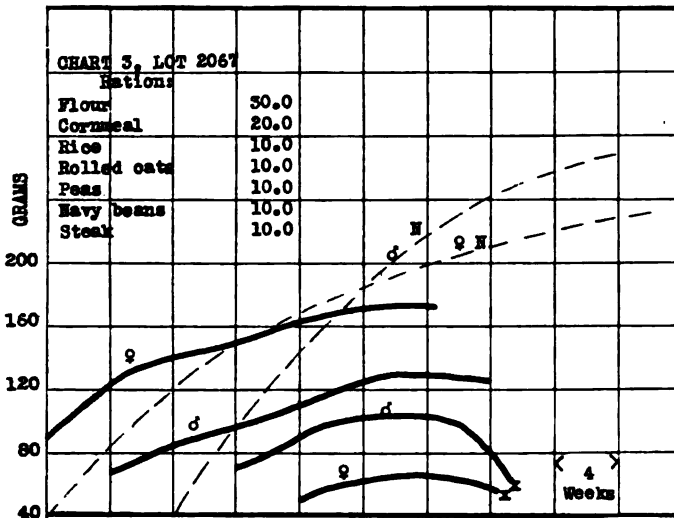
The animals confined to this diet failed to grow, but in most cases were able to maintain their initial body weight for a period of nearly 4 months. The condition of the skin remained normal.

There was none of the roughness or soreness which we have frequently seen in rats long confined to certain types of faulty diets (3). They became thin and frail, but presented none of the symptoms which were observed by Chittenden and Underhill in dogs as the result of restricting them to a diet of peas, crackers, and cottonseed oil. That this diet is deficient only in respect to well recognized dietary factors is shown by the behavior of Lot 2047, which was fed essentially the same mixture supplemented with purified protein, inorganic salts, and butter fat (fat-soluble A). With these additions the diet is rendered sufficiently complete to make it possible for young rats to grow at nearly the normal rate to what may be regarded as but little below the normal adult size, and to produce young. They were not able to rear their young, and cannot, therefore, be considered to be in an entirely normal state of nutrition.

Lot 2047 shows the growth curves of a group of young rats which were fed a diet which was identical with that of Lot 2199, Chart 1, except that the former was supplemented with purified protein, inorganic salts, and fat-soluble A. These additions rendered the diet very nearly complete, since the animals were able to grow at a good rate and to produce young. They remained somewhat undersized in all cases. Although one female remained sterile, the other produced two litters of young but was unable to rear them. It is not entirely clear what was the limiting factor in causing these animals to fall below satisfactory nutrition. It may well be that the preponderance of products of the endosperm of seeds made the phosphorus content of the diet too low. We have not yet determined whether this is the case. It is also probable that when such a high percentage of the diet is derived from degerminated and decorticated seed products, starch, sugar, and molasses, the content of the dietary factor water-soluble B is near the actual amount required for the maintenance of growth, and is below the amount necessary to promote optimum well being. All animals were supplied with iron in the drinking water, and once each week with iodine, according to the usual routine procedure in our colony.

Chart 3.—Lot 2067 shows the slow rate of growth and early stunting of young rats which were fed a diet containing the entire substance of three kinds of seeds, *viz.*, peas, rolled oats, and navy

beans; the portion representing the endosperm of three seeds (bolted wheat flour, degerminated corn-meal, and polished rice), together with 10 per cent of the solids of the diet derived from round steak. This diet furnished 19.4 per cent of protein, wide variety of origin, palatability in high degree, and was derived from both animal and vegetable foods, yet it was not of a character suitable for the maintenance of satisfactory nutrition during growth, nor did it prevent the early aging of the animals. The animals in this group were very old and dirty looking at the age of 6 months. These results emphasize the point to which we have called atten-



tion on several occasions; viz., that all the foods which were included in this diet are faulty in respect to two factors, one of which is the lack of sufficient calcium, sodium, and chlorine, the other a lack of a sufficient amount of the dietary essential fat-soluble A. These faults can be corrected by the inclusion in the diet of a sufficient amount of milk and eggs (see Chart 5, Lot 2148), and to some extent by the amounts of leafy vegetables which can be eaten by man. Eggs correct all faults in any diet except with respect to calcium, which element is not abundant in the contents of the shell. The growing chick derives a considerable amount of lime from the shell during the period of incubation.

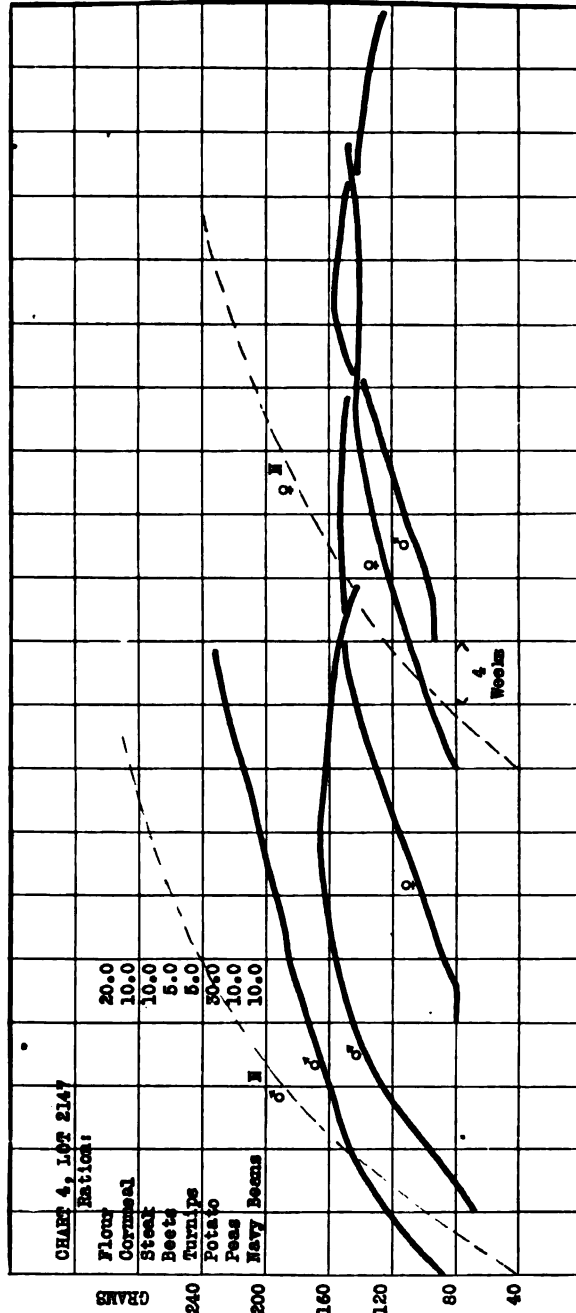


CHART 4.

Chart 4.—Lot 2147 illustrates the growth of a group of young rats which were fed a diet consisting of two endosperm products (wheat flour and degerminated corn-meal), two edible roots (beets and turnips), one tuber (potato), two legume seeds (peas and navy beans), and 10 per cent of the dry matter of the diet as cooked, dry round steak. The important thing to note about this food mixture is that it is derived entirely from storage organs of plants or products made from them, together with muscle tissue. The latter is a highly specialized tissue, which does not manifest metabolic functions comparable with the glandular organs. Muscle tissue resembles the seeds in its dietary properties except that its proteins are of better quality. It does not supplement foodstuffs of the storage organ group except with respect to protein, and accordingly such a diet as is here described is deficient in two respects, *viz.*, in calcium, sodium, and chlorine among the inorganic elements and with respect to fat-soluble A.

These rats are too short and broad to be considered normal. They had begun to look old at the end of 160 days on this diet, and at the end of 10 months they were very thin haired and aged looking, with very scaly, sore ears and tails. The eyes have begun to show signs of inflammation and apparently they are on the verge of developing xerophthalmia.

A dinner consisting of steak, bread made without milk, butter, potatoes, peas or beans, gravy, a flavored gelatin dessert, and coffee, may appear to constitute a satisfactory meal, for it affords variety, palatability, and meets the requirements of the food chemist or the dietitian, but it would not promote health in an experimental animal over a very long period if taken as a monotonous food supply.

Chart 5.—Lot 2148 is of special interest when compared with Lot 2147, Chart 4. Their rations were closely similar, consisting of cereal products, legumes, tubers, and edible roots, but the ration of Lot 2148 contained 10 per cent of dried whole milk (Merrill-Soule). A comparison of the growth curves shows how marked is the tendency of milk to correct the deficiencies of foods of the storage organ group of vegetable origin, and likewise of muscle tissue.

These animals were able to grow at the normal rate to the full adult size, except one individual. Reproduction was distinctly

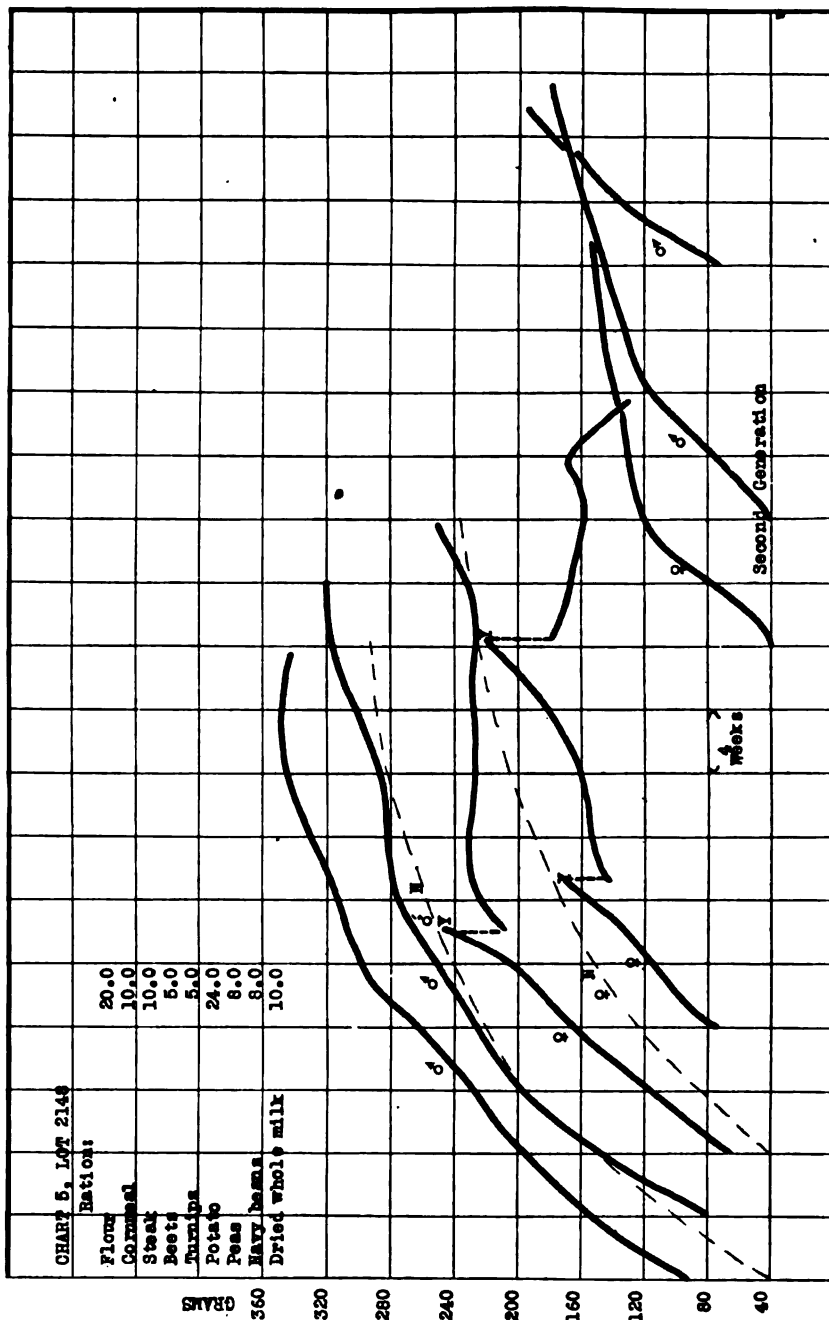


CHART 5.

below normal in amount, but the young which were born were reared with a fair degree of success. That the diet is somewhat faulty is shown by the reproduction records, and by the behavior of the second generation when confined to the diet of the parents. We have not yet determined the reason for the failure of 10 per cent of milk to supplement the remainder of the diet completely. It seems almost certain, however, that it is due to the failure of the amount of milk added, although liberal, to supply enough calcium. We have been able to relieve xerophthalmia in rats by feeding the fat isolated from evaporated milk (4), and have convinced ourselves that the fat of dried whole milk still contains a considerable amount of fat-soluble A, so a shortage of fat-soluble A cannot be the limiting factor in this case. We know that the proteins of this diet are of excellent quality, and the content of protein amounted to 18 per cent of the food mixture, which is an ample amount. Iron was furnished in the drinking water, and is abundant in red meats, and since the fault must be in the inorganic moiety, it is most probably calcium.

Chart 6.—Lot 2152 shows that a diet derived from the entire substance of three seeds together with polished rice, degerminated corn-meal, and bolted wheat flour, supplemented with calcium carbonate and sodium chloride, induced better growth than did the same seed products supplemented with 10 per cent of cooked, dried round steak (see Chart 3). It is evident that the lack of these salts is a matter of greater importance than is the quality of the protein, or the relative shortage of fat-soluble A. Meat enhances such a grain mixture only with respect to the improvement of the quality of the protein (see Chart 7).

Although the two females in the group produced two litters of young each, none were satisfactorily nourished. One female had a litter of nine which at 16 days weighed 117 gm. The number of young was reduced at that time to five. Four of these reached an age of 45 days at which time they weighed collectively 103 gm. Although very small for their age, they were active. A litter from another mother (six young) weighed 98 gm. at 19 days of age. Three reached the age of 34 days when they weighed 67 gm. together. Two reached the age of 69 days. They were then very puny and weighed together but 73 gm. They should have weighed at least 100 gm. each at that age. (Compare these repro-

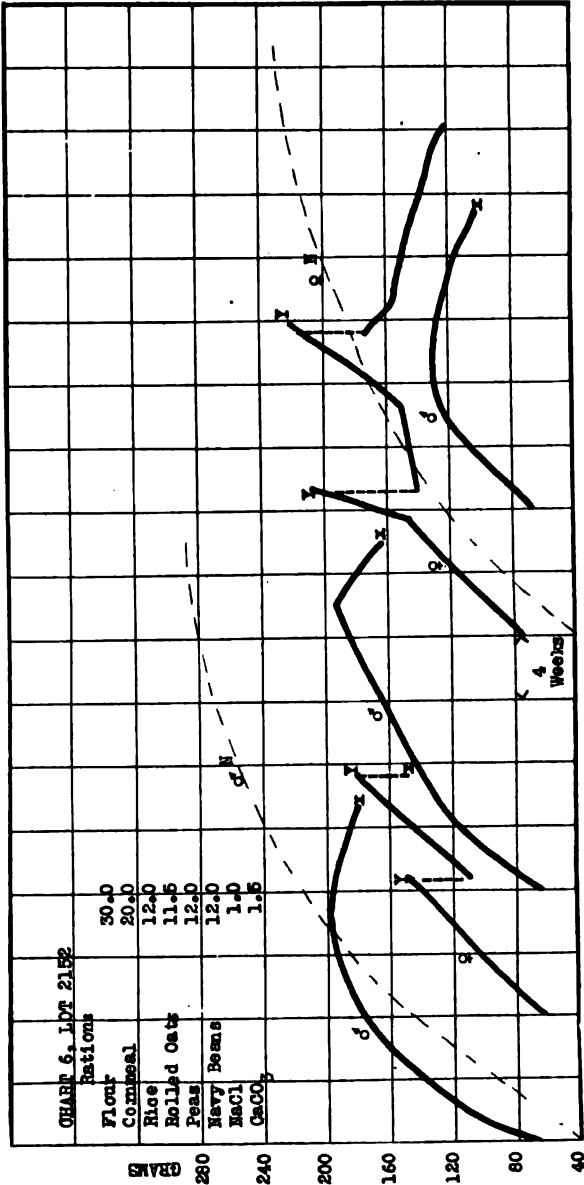


CHART 6.

duction records with those of Charts 7, 9, 10.) At the age of 5 to 6 months the rats restricted to this diet were very old looking. Even with diets derived from wholesome natural foodstuffs, and having such a composition as will comply with the standards of the food chemist, the source of the several components of the food supply is a matter of the first importance, and is one of the principal factors in determining the span of life and vigor of an animal (compare with Chart 3, Lot 2067).

Chart 7.—Lot 2153 received a mixture which was similar in all respects to the ration of Lot 2067, Chart 3, but supplemented with sodium chloride and calcium carbonate. Thus supplemented, the mixture of seeds and seed products with 10 per cent of beef-steak supported good growth to the full adult size, and the females produced about the number of young which we should expect of well nourished rats. Two first generation females produced six litters (forty-four young), of which thirty-five were successfully weaned. The second generation did not do so well with their young, although the diet enabled them to grow up fairly well.

Although the young of the original experimental group were apparently in excellent condition, they were actually not so. One of these second generation females died at the age of $4\frac{1}{2}$ months without having had young. Another grew up in a nearly normal way, had a litter of young at the age of 129 days, but they were all dead, and the mother died a month later. A third female appeared normal as she reached maturity, and had a litter of young when she was 87 days old. The young were small and puny and were eaten by the mother. The diet sufficed for the maintenance of the original group during the growing period, and supported fair vigor during reproduction, but there were sufficient faults in its make-up to undermine the vitality of the offspring. Such records as these convince us that we have now refined our experimental technique so as to make possible the detection of very slight faults in the diet.

The practice of indulging in a variety of foodstuffs, and of alternating among certain of them instead of restricting the diet to a monotonous mixture is without doubt a highly desirable one, but where the selection of food is limited too largely to that group of products which are functionally storage tissues of plants and to meats (muscle tissue) variety within this range will not prevent deterioration of the race.

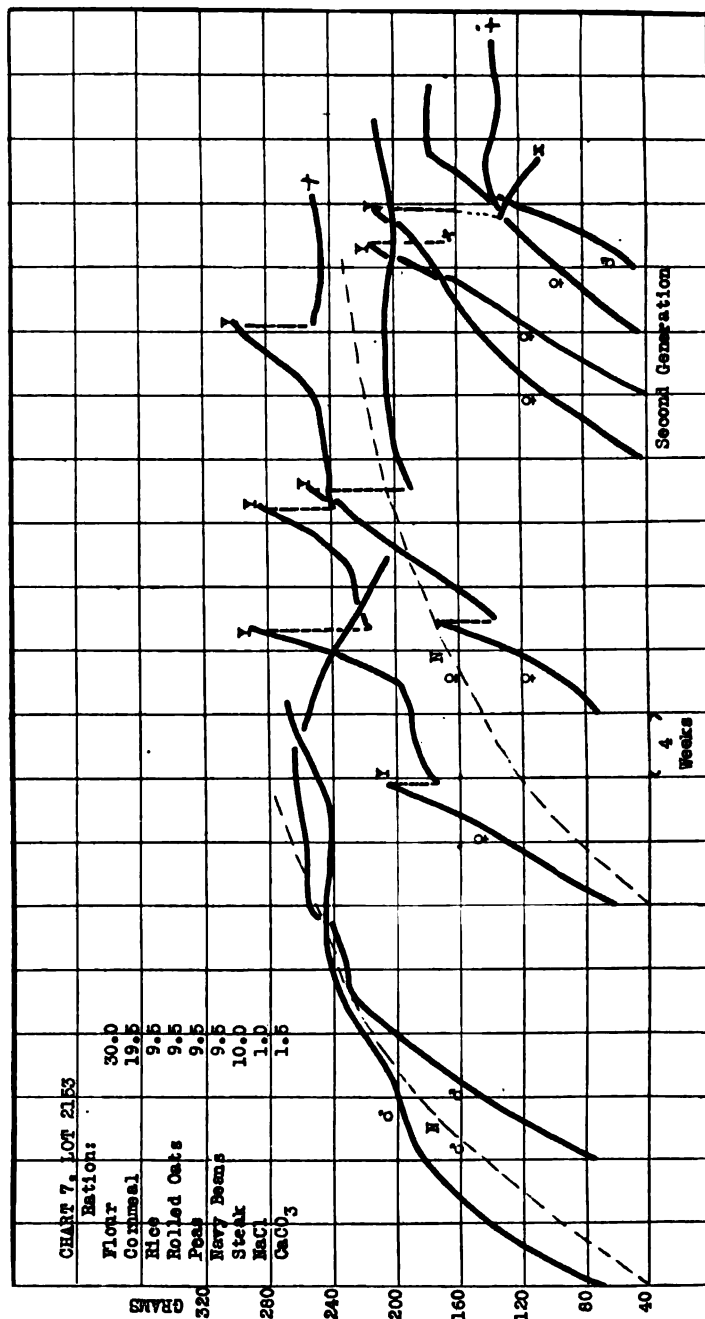


CHART 7.

Chart 8.—The records of Lot 2064 show to what degree a content of cabbage leaf equivalent to 10 per cent of the diet supplements a mixture of wheat flour, corn-meal, potato, peas, navy beans, and beefsteak. Without the presence of the leaf in this diet very little growth could be secured. With 10 per cent of cabbage growth could take place, but the ultimate size attained was not much greater than half the normal adult size. Two females each had a litter (six and seven young respectively) of which eleven

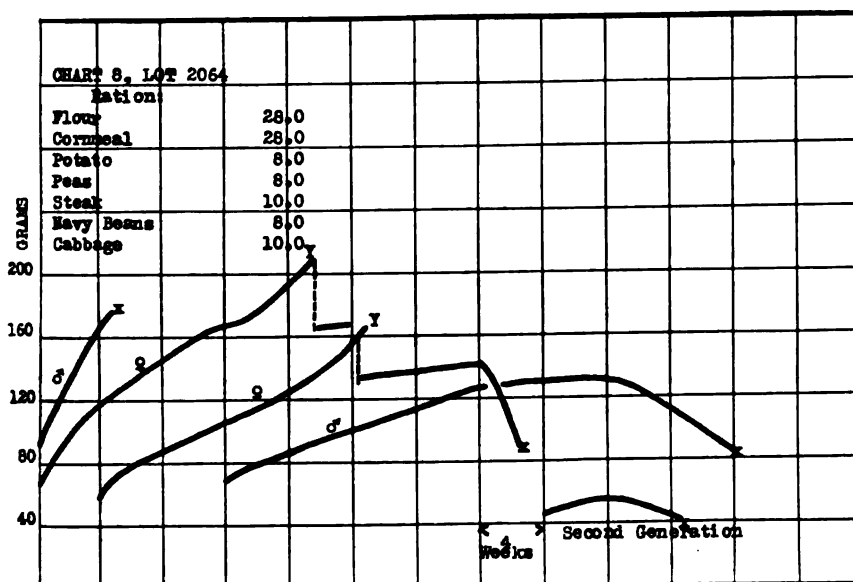


CHART 8.

were weaned but none of them was normal. These were too short, an appearance which is characteristic of many young rats in our colony which become somewhat deformed because of malnutrition. We are studying this condition closer. They were very nervous and undersized. Five young in one litter weighed 204 gm. at the age of 49 days. Six young in the other litter weighed 187 gm. at the age of 46 days. We have seen a litter of four well nourished young rats reach a collective weight of 300 gm. at the age of 35 days.

When the rats on this ration had been confined to the diet 5½ months, they were already very old and rough, and their condition grew worse rapidly, none living beyond the age of 8 months. The offspring were unable to grow when confined to the diet of their parents.

In order to appreciate the extent to which the addition of moderate amounts of leaf enhances a diet derived from seeds, tubers, roots, and meats, Lot 2064 should be compared with Charts 3 and 4. A comparison of Charts 3 and 4 with 6 and 7 illustrates the good effects of supplementing this type of diet with sodium chloride and calcium carbonate. A comparison of the physiological results of feeding a diet derived from products belonging to the storage organ group in plants and the same mixture supplemented with milk is seen in Charts 4 and 5. Chart 7 shows, when compared with Chart 8, that the correction of the inorganic factor is one of the chief values of the inclusion of a leaf in the diet. That the addition of leaf enhances a diet of seeds, meat, and the necessary salts is shown by a comparison of Charts 7 and 9, and the desirability of having milk, a leaf, and likewise additions of sodium chloride and calcium carbonate is shown by a comparison of Charts 9 and 10.

Chart 9.—The ration of Lot 2069 was similar to that of Lot 2148, Chart 5, except that milk was replaced by 10 per cent of dried cabbage and sodium chloride and calcium carbonate. These animals grew well and were very prolific. Two females produced seven litters (thirty-six young) up to the age of 8½ months. This we regard as normal fertility. Of the thirty-six young by these two mothers twenty-eight were successfully weaned. A daughter of one of them, when confined to the diet of the group, has had four litters (twenty-one young) at the age of 259 days. The first litter was born when she was but 71 days old, which shows that she reached maturity early. She reared thirteen of the twenty-one young, but these were too short for their age and weight, a condition frequently seen in stunted rats. One third generation female (granddaughter of the first group) had her first litter of four young at the age of 94 days. These weighed collectively 202 gm. at the age of 28 days, a weight which is highly satisfactory. At the age of 11 months the original group look very old and rough, and are distinctly poorer in condition than is Lot 2070,

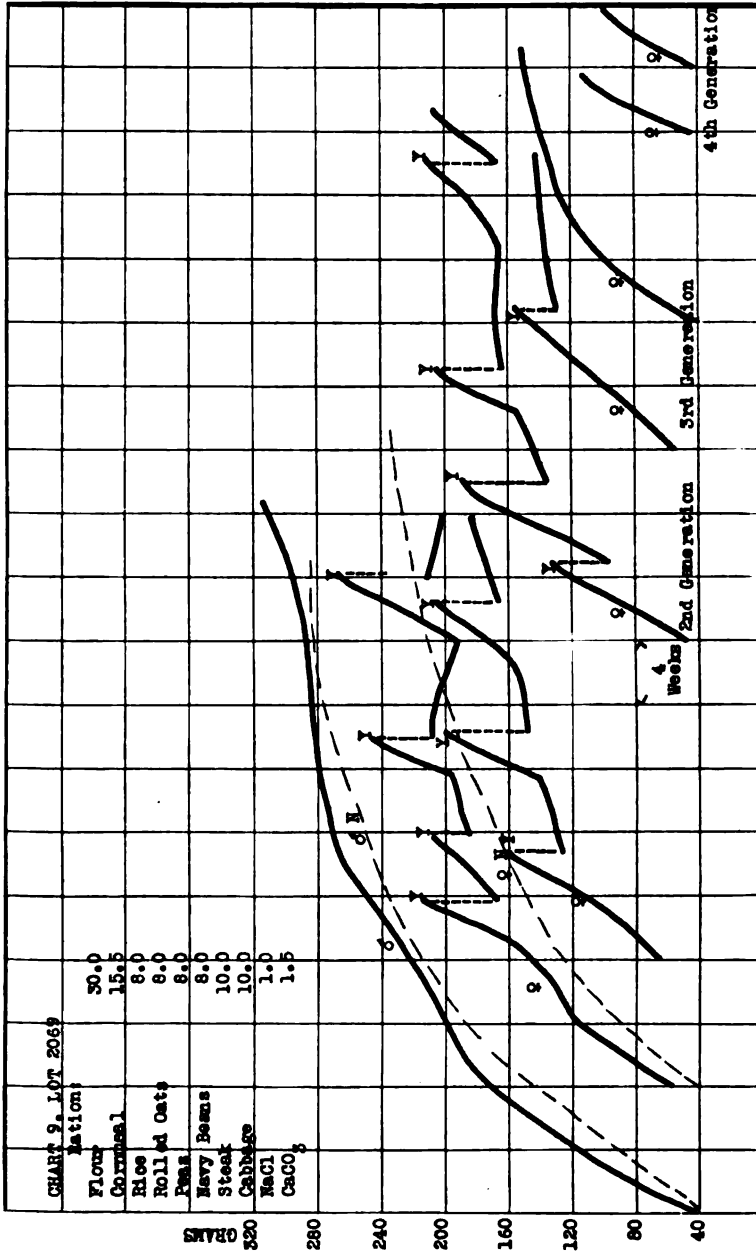


CHART 9.

Chart 10, whose diet is similar, but contains 5 per cent of whole milk powder.

This data reveals the profound importance of a knowledge of the dietary properties of our natural foodstuffs and the combinations which have efficient supplementary relations from the dietary standpoint. This is very nearly the same type of diet which is common among many Oriental peoples, with the differences of course that they have more or less variety in the leafy vegetables which they eat, but restrict themselves to rice as the principal cereal, employ soy beans instead of navy beans, and use more fish than flesh of mammals, especially among the poor. The important thing to appreciate is the classification of their foods and ours on the basis of their biological function. The dietary properties can then, in the light of our studies with several natural foods, be interpreted in a manner that surpasses the most sanguine expectations of the investigator of a few years ago.

It should be kept in mind that the leaf is not the equivalent of milk as a supplementary and corrective food for diets largely made up of degerminated and decorticated cereal products, peas, beans, rolled oats, and steak. The leafy vegetables are highly desirable additions to the diet and their use should be considerably extended in this country, but they are not sufficiently acceptable to the palate of man to make possible the consumption of a sufficient amount of this class of foods except by one who is schooled, as are many of the poor among the Orientals, in eating for the sake of maintaining life, rather than for the pleasure of tasting a great variety of dishes which are the creation of geniuses in the culinary art.

Chart 10.—Lot 2070 illustrates the growth and reproduction records of a group of rats which was fed a similar diet to Lot 2148, Chart 5, and Lot 2069, Chart 9, but with the mixture of foodstuffs derived from the storage tissues of plants, and the muscle tissue of animals supplemented with both 10 per cent of dried cabbage and 5 per cent of dried whole milk, and sodium chloride and calcium carbonate. Even when a diet derived from seed products and meat is supplemented with liberal amounts of leaf, which improves its quality in some degree, it still falls far short of the optimum, as is shown by the great improvement in vitality and ability to meet the more strenuous demands of reproduction, to escape

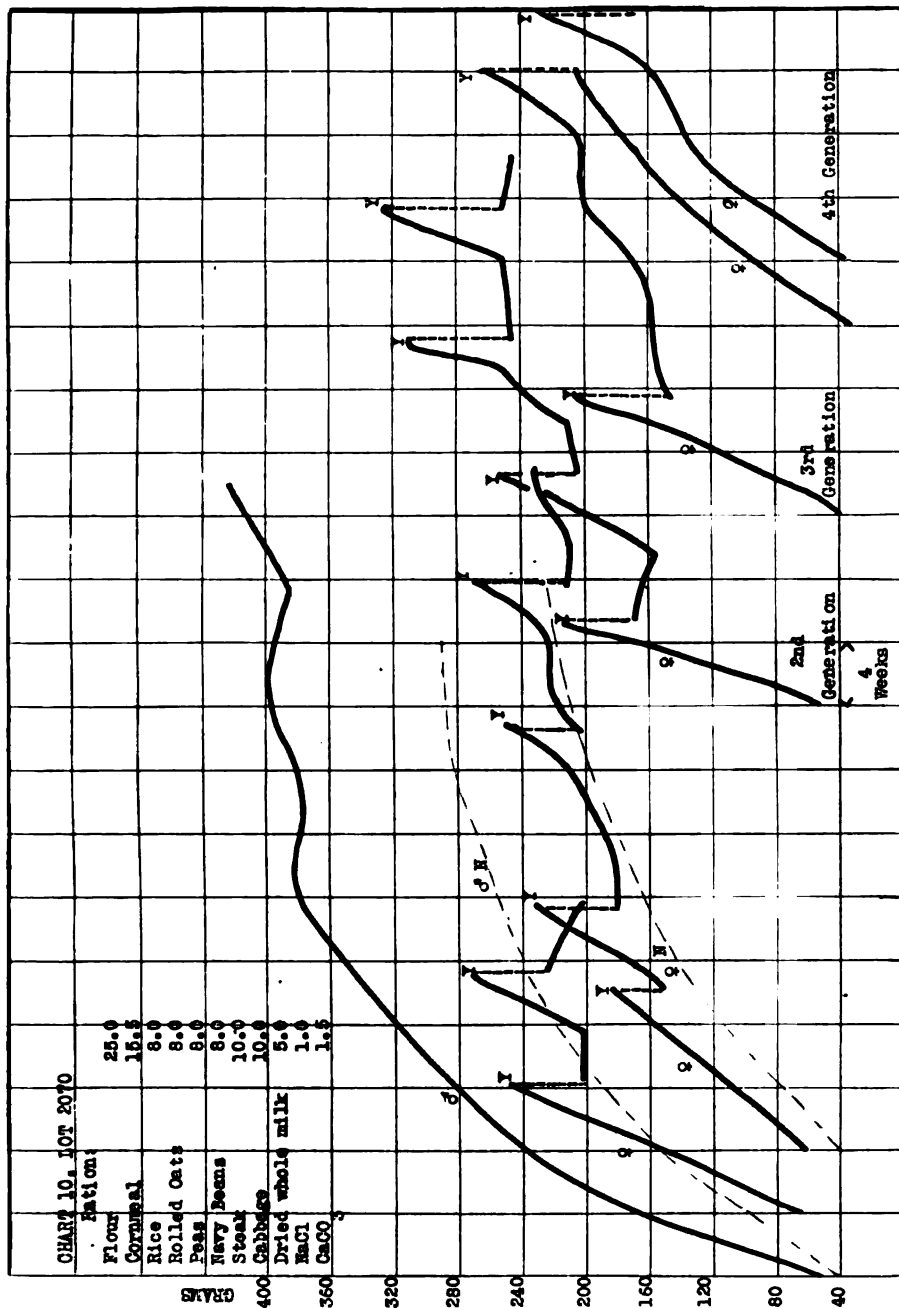


CHART 10.

lung infection, and to maintain to an advanced age the vigor of middle life. Such improvement can be secured by the liberal use of milk with diets of this type.

The reproduction records of these rats are of special interest. Those in Lot 2069, Chart 9, had an infant mortality of 22 per cent in the first generation on the diet, and the female from this group of young which grew up on the same diet lost 38 per cent of her twenty-one young. It is very rare indeed that a young rat is not successfully weaned in our breeding stock.

An idea of the infant mortality in well nourished rats can be gained from the records of Lot 2070. In the original group in Lot 2070 the two females up to the age of 339 days had seven litters (forty-eight young) of which forty-seven were successfully weaned. Two females from these young which grew to maturity had up to the age of 284 days six litters (50 young) of which forty-eight were weaned. A granddaughter of the original group (*i.e.*, one of the third generation on the diet) had up to the age of 259 days had two litters (17 young) of which all were weaned successfully. Two great granddaughters each weaned successfully a litter of 8 young.

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EXPLANATION OF PLATE 1.

FIG. 1. Female rat from Lot 2147, Chart 4. The diet of this group consisted of wheat flour 20, degerminated corn 10, cooked, dried potato 30, peas 10, navy beans 10, beets 5, turnips 5, and cooked, dried beefsteak 10 per cent.

This diet was derived like that of Lot 2148 from degerminated cereal products, legume seeds, tubers, edible roots, and meat (muscle tissue), but differed from it in not containing milk. It afforded wide variety, had an appropriate chemical composition in so far as analysis could show, was palatable, and included only natural food products of recognized wholesomeness, and from both animal and vegetable sources. Notwithstanding these facts the nutrition of the animals restricted to this food supply was very faulty. The photograph shows a rat at the age of 308 days, or at about the end of the first quarter of the normal span of life for a well nourished animal of this species. It weighed but 137 gm. as compared with the one pictured from Lot 2148, whose weight was 245 gm. The pronounced stunting and very rapid aging of this group emphasize the importance of including in the diet the foodstuffs which we have designated as "protective foods." Milk is the most effective of these.

FIG. 2. Female rat from Lot 2148, Chart 5. The diet of this group consisted of bolted wheat flour 20, degerminated corn-meal 10, potato 24, peas 8, navy beans 8, turnips 5, beets 5, beefsteak 10, and dry whole milk (Merrill-Soule) 10 per cent.

This diet was derived from the same foodstuffs as that of Lot 2147, and in closely similar amounts, and differed from it only in containing 10 per cent of its dry substance as dry whole milk. The addition of this amount of milk corrected the faults in the remainder of the diet in a manner that is most surprising (see Charts 4 and 5). The animal was 308 days old and weighed 245 gm. when photographed. It was, therefore, but little beyond one-fourth through the normal span of life for the species.



FIG. 1.



FIG. 2.

(McCollum, Simmonds, and Parsons: Pellagra-Producing Diets. VI.)

IS LACTALBUMIN A COMPLETE PROTEIN FOR GROWTH?

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(From the Research Laboratory of Parke, Davis and Company, Detroit.)

(Received for publication, March 24, 1919.)

Recent reports of investigations suggest that there is a difference of opinion as to the growth-promoting value of lactalbumin. We are especially interested (1) in this point since many of our findings as to the relative value of certain vitamine preparations, when added to an otherwise complete diet, have been based upon the assumption that our basal protein, lactalbumin, is capable of promoting growth; that is, that it contains the fundamentals for furnishing *all* the protein complexes required.

Osborne and Mendel (2) have shown very clearly that they were able under their conditions to obtain excellent results with lactalbumin as the primary protein in their diets. In fact, they (3) found in comparing the food value of edestin, casein, and lactalbumin for the growth of young rats that "the superior efficiency of the lactalbumin in the nutrition of growth is demonstrated beyond question." "Thus, to produce the same gain in body weight, 50 per cent more casein than lactalbumin was required, and of edestin nearly 90 per cent more." In a recent article, Osborne, Mendel, and Ferry (4) suggested a method of calculating the growth-promoting value of various proteins in rations. This method was based upon correlating the gain in body weight with the grams of both total food and protein consumed. When the authors compared their results for casein and lactalbumin on this basis, they found that the same gains for these two proteins could be made, over the same length of time, only when the casein-fed rats consumed 20 and 24 per cent more of ration and protein respectively than did the lactalbumin-fed rats.

On the other hand, McCollum, Simmonds, and Parsons (5) have taken the stand that lactalbumin is an incomplete and poor

protein for growing rats. Their conclusion was based upon the fact that when they compared casein with lactalbumin in two rations which were alike in all other respects, normal growth resulted in the first instance and almost no growth in the latter instance. Their basal diet was made up of peas 45 per cent, salt mixture 1.8 per cent, agar-agar 1 per cent, dextrin 38 per cent, and butter fat 5.0 per cent. 9 per cent of lactalbumin and of casein was added, respectively, to two portions of this basal ration, bringing the total protein up to about 18 per cent. The Osborne and Mendel rations consisted of 18 per cent protein (lactalbumin or casein), 28 per cent protein-free milk, 28 per cent starch, 18 per cent butter fat, and 10 per cent lard. McCollum and his associates stated:

"We are forced to the conclusion that lactalbumin is a poorly constituted or an incomplete protein and that the excellent results of Osborne and Mendel were due to the high proportion of nitrogen derived from the 'protein-free milk' which was present in their food mixtures and served to supplement the lactalbumin with respect to some as yet undetermined cleavage product which is essential for growth."

According to Osborne and Mendel, their protein-free milk was made up of 80 per cent lactose, 15 per cent inorganic salts, 2.2 per cent protein, 2.7 per cent non-protein matter, and carried the water-soluble B vitamins. Mitchell and Nelson (6) found that they could reduce the per cent of protein nitrogen in the "protein-free milk" by neutralizing the boiling milk whey; but by this procedure they contaminated the lactalbumin, since calcium phosphates were thrown down at the same time. Kennedy (7) has shown that the Osborne and Mendel protein-free milk contains "either unprecipitated protein or peptides of considerable size;" that about half of the nitrogen was precipitated by certain salts in the non-amino form, and that after tryptic digestion, there was an increase in amino nitrogen.

From the above facts, it is evident that protein-free milk is a complex mixture and possibly capable of carrying the proper kind of protein or nitrogenous cleavage products to furnish the nitrogenous supplement that may be needed for an incomplete protein. Whether there would be a sufficient *amount* of this nitrogenous supplement present is another question.

DISCUSSION.

In using lactalbumin as the sole protein in the diet of young rats and as a supplement to an incomplete protein for growth, we have obtained results which agree with Osborne and Mendel (Charts 1, 2, and 3). On the other hand, we have recorded data with lactalbumin which correspond to what McCollum, Simmonds, and Parsons found (Chart 4). That is, we have secured in many cases normal growth, and then again, in using the same lactalbumin in the same amount, we have obtained almost no growth at all. However, the rations differed in this respect—when protein-free milk was present we obtained growth, but when a vitamine extract, salt mixture, and starch were substituted for the protein-free milk, poor growth resulted. In other words, our results agreed with what McCollum and his associates claimed; *i.e.*, the protein-free milk was responsible for supplying the needed accessory for the lactalbumin. *Our results do not show that the nitrogenous matter in this protein-free milk played the fundamental part in furnishing the necessary supplement.*

According to Osborne and Mendel, the protein of their protein-free milk constituted about 0.13 per cent of the food. Mitchell and Nelson claimed that the reduction in nitrogen which they were able to effect was due to protein. If this is so, their procedure gave a still lower protein content than that of Osborne and Mendel, bringing it down to about 0.08 per cent of the ration. Such small amounts of protein as these would hardly seem to be sufficient to supply enough cleavage products to cause the difference that McCollum and his associates found between casein and lactalbumin, or that we have found (Charts 1 and 4). Our lactalbumin was separated out, in accordance with the method of Osborne and Mendel. It was, however, thoroughly digested with hot alcohol a number of times, and finally with ether or acetone. In this way, we considered that all traces of the water-soluble B and fat-soluble A vitamins were removed.

With the idea of determining whether this growth-promoting accessory factor was specific for the protein-free milk when lactalbumin was the sole protein, various rations were fed which differed only in respect to the kind of synthetic protein-free milk that was used. These were made up of the McCollum (8) salt

mixture 185, an equivalent amount of lactose that had been purified by repeatedly extracting a pure product with hot 95 per cent alcohol and drying in vacuum at a low temperature, purified starch, and an extract containing the water-soluble B vitamine. The extracts used were: a cold water digest of wheat germ; a hot alcohol (95 per cent) digest of wheat germ that had been previously extracted with ether to remove the oil; a cold alcohol (95 per cent) digest of dried brewers' yeast; or a water-soluble fraction of autolyzed brewers' yeast.

Chart 5 illustrates the growth-promoting property of these rations where lactalbumin was the sole protein and no protein-free milk was present, and shows that normal growth is possible. In the case of the water extract of wheat germ, which carried the vitamine B, the amount of solids used was based upon the nitrogen content, 5 per cent yielding 0.11 gm. of nitrogen per 100 gm. of ration. The hot alcohol (95 per cent) extract of the wheat germ carried 0.08 gm. of nitrogen per 100 gm. of ration. And the cold alcohol extract of dried brewers' yeast added 0.04 gm. of nitrogen per 100 gm. of ration. In the case of the hot alcohol extract of wheat germ and the cold alcohol extract of dried yeast, the amount of protein nitrogen must have been very low, if in fact any was present. Therefore, the amount of nitrogen that these two extracts added to the rations was in the non-protein form.

It would seem then, if lactalbumin were in reality an incomplete protein for growth, that the various extracts, furnishing the water-soluble B vitamine, carried the same non-protein cleavage accessory that the protein-free milk supplied. Further, the protein nitrogen, in the protein-free milk and in the water extract of wheat germ, was not concerned in supplementing the growth-promoting property of the lactalbumin.

McCollum and Davis (9) found that lactose and casein, unless thoroughly purified, seemed to carry enough water-soluble B and fat-soluble A accessories or vitamines to cause fairly good growth when these nutrients were added to a diet that was otherwise complete, except for these food hormones. Drummond (10) has confirmed their work. It might be contended, therefore, that these two accessory substances were adsorbed by our purified lactose and that they were responsible for the growth which we

obtained with the lactalbumin supplemented with the extracts of wheat germ and yeast.

Since both Osborne and Mendel, and McCollum and his associates used butter fat as their source of the fat-soluble A, we would be concerned only as to whether the *kind* or *source* of water-soluble B in the lactose was enough different from that in the wheat germ or yeast to be the contributing factor. The curves in Chart 6 show clearly that our lactose lacked a sufficient amount of the water-soluble B vitamine to promote growth. Thus, when this lactose and the lactalbumin were incorporated in rations, made up according to the Osborne and Mendel (18 per cent butter fat and 10 per cent lard) and the McCollum (5 per cent butter fat) formulas, the rats soon stopped growing and showed the usual symptoms which accompany a lack of this accessory in the diet.

As stated above, we have used rations which contained lactalbumin as the sole protein and which we considered from all the known facts obtainable to be abundantly capable of producing normal growth in rats. Yet some of these food mixtures gave poor growth (Chart 4) just as McCollum, Simmonds, and Parsons found with lactalbumin in their modified diet of peas, and as Osborne and Mendel (11) found when they compared the value of casein, lactalbumin, and edestin supplemented with their artificial protein-free milk and dried yeast. In these cases we did not use protein-free milk or lactose. When the rations were modified so that a part of the starch was replaced by an amount of purified lactose equivalent to that present in 28 per cent of protein-free milk, the rats began to grow normally (Chart 7).

From the evidence that we have offered, it would appear that the reason we were unable, in some cases (Chart 4), to obtain anything like good growth with lactalbumin as the sole protein, was due to the absence of lactose in the diets. Aside from this fact, it seems that the protein-free milk (which contained 80 per cent lactose) furnished nothing essential that was not supplied by the alcoholic extract of wheat germ or yeast, together with the synthetic salt mixture and lactose. Therefore, we do not agree with McCollum, Simmonds, and Parsons that the high nitrogen content of the protein-free milk contributed some cleavage product which adequately supplemented what they claimed to be incomplete lactalbumin. *Rather, we take the position that the lactalbumin*

is a complete protein in the sense that it is not lacking in any essential nitrogenous cleavage product for growth.

We are inclined to express the following tentative explanations, based upon our data, as to why lactalbumin does not produce normal growth unless supplemented by lactose in the form that we used it. First, the lactalbumin protein molecule may be so constituted that it is easily susceptible to toxic or inhibitory substances, while the casein protein molecule may be more stable or resistant. On this basis, we would say that in the wheat germ and yeast extracts, which carried the water-soluble B accessory, there may have been some toxic substance present which prevented normal growth with the lactalbumin until the lactose was introduced. With the modified lactalbumin-peas diet of McCollum, Simmonds, and Parsons, (5) there was also present a toxic substance which acted exactly as it did in our diets when no lactose was present, but in the casein-peas diet, the toxicity factor did not manifest itself.

Second, in the separation of the casein, the lactalbumin, and the protein-free milk from fresh skimmed milk, the lactose (and possibly the casein) adsorbs not only the water-soluble B and fat-soluble A accessories, as McCollum and Davis, and Drummond have shown, but it (and the casein) may *also* adsorb another as yet undefined water-soluble vitamine. In separating the lactalbumin, this accessory does not seem to be adsorbed, or if it is, the vitamine is split off much more easily during the subsequent washing and purification than is the case with lactose (or casein).

Applying this last suggestion, we would say that our purified lactose still had this undefined water-soluble vitamine adsorbed to it; on the other hand, that the casein of McCollum and associates was the carrier of this accessory; while in the feeding trials of Osborne and Mendel that this vitamine was present in their protein-free milk (2) but absent in their artificial protein-free milk without lactose (11). To substantiate this deduction regarding casein, we refer to the work of Funk and Macallum (12) who found that this protein, even though it was purified by the method which McCollum (9) uses, carried an accessory which they say may be analogous to the antiscorbutic vitamine.

It seems plausible to conclude from these data that the second explanation is the more probable, although it is easy to conceive

that both the above suggestions may be needed at times to explain the nutritive value of the lactalbumin. It is, therefore, difficult, in the light of these results, to agree with McCollum, Simmonds, and Parsons that the protein-free milk carried enough *nitrogenous* cleavage products to supplement what they designate as the incomplete and deficient growth-promoting lactalbumin, and yet when this protein-free milk supplemented the casein, it contributed nothing to accelerate the rate of growth of this protein and make it even the equal of the lactalbumin. If their conclusion was correct, how can one explain why Osborne and Mendel (2) obtained such contrasting results as to the relative growth-promoting values of lactalbumin, casein, and edestin when these proteins were incorporated in rations with the same amount of protein-free milk present in each (13)?

CONCLUSIONS.

1. Lactalbumin is a complete protein in the sense that it does not lack any of the nitrogenous cleavage products essential for growth. It can supplement a deficient growth-promoting protein (corn gluten) and, incorporated as the sole protein in a ration containing lactose, it produces a normal rate of growth, when present to the extent of only 10 per cent.

2. Lactalbumin is a protein which either is sensitive to certain toxic substances and under these conditions growth is retarded unless adequate adjustment is made in the diet; or it is a protein which does not appear to be able to adsorb what we tentatively designate as a vitamine (other than water-soluble B).

3. Lactose seems to be the essential constituent in the protein-free milk that contributes the accessory which makes an otherwise incomplete lactalbumin diet bring about normal growth in rats.

4. Lactose, when added to a lactalbumin diet, either may have the physiological property of overcoming the toxicity that inhibits growth taking place, or else it may carry a water-soluble vitamine (other than the water-soluble B) which appears to be essential to growth. This phase of the study is being continued.

The authors wish to express their appreciation to Lieutenant M. E. Slater for valuable assistance in the preliminary work of this

study. He was obliged to sever his connection with this work at an early stage as he was called into military service. To Mr. Charles Hunter also our thanks are due for aiding in the routine work.

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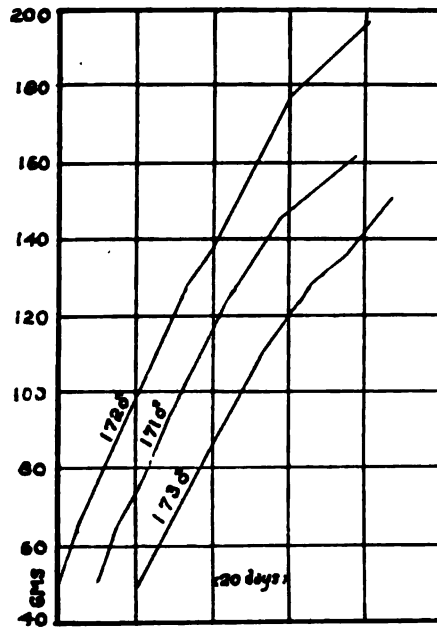


CHART 1. Good growth is obtained on a diet of 10 per cent lactalbumin protein, 28 per cent of Osborne and Mendel protein-free milk, 18 per cent butter fat, 10 per cent lard, and starch.

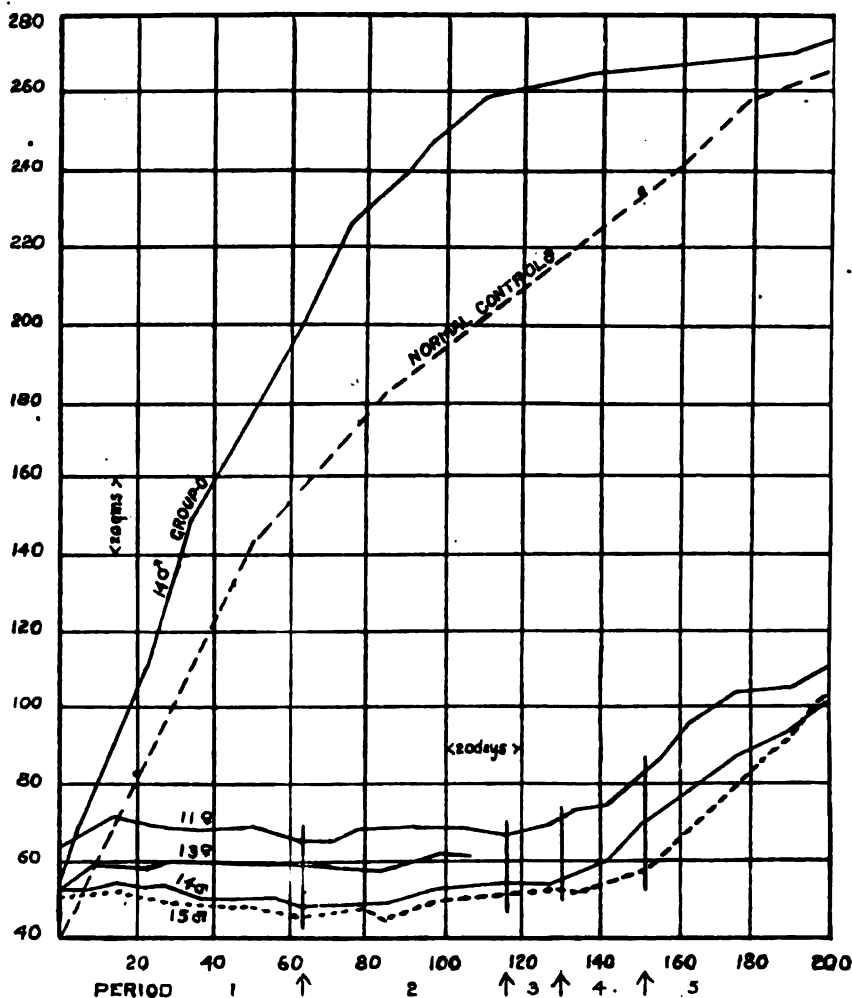


CHART 2. The curves illustrate in the case of Rat 14, Group O, the excellent growth response that can be obtained over a long period of time with the same diet that was fed to the groups of rats represented in Chart 1.

The curves also show, in the case of Rats 11, 13, 14, and 15, an interesting cycle of growth. Thus, throughout, the basal diet was 28 per cent of the Osborne and Mendel protein-free milk, 18 per cent butter fat, 10 per cent lard, and starch to make up the balance. In Period 1 (maintenance) there was 7 per cent corn gluten protein; in Period 2 (maintenance) 10 per cent corn gluten protein; in Period 3 (slight growth) 7 per cent protein of which 28 per cent was lactalbumin and 72 per cent corn gluten; in Period 4 (growth) 10 per cent protein with 18 per cent of it lactalbumin and 82 per cent corn gluten; and in Period 5 (growth) 10 per cent protein of which 32 per cent was lactalbumin and 68 per cent corn gluten. There can be no doubt but that the lactalbumin is capable of supplementing the growth-deficient corn gluten protein.

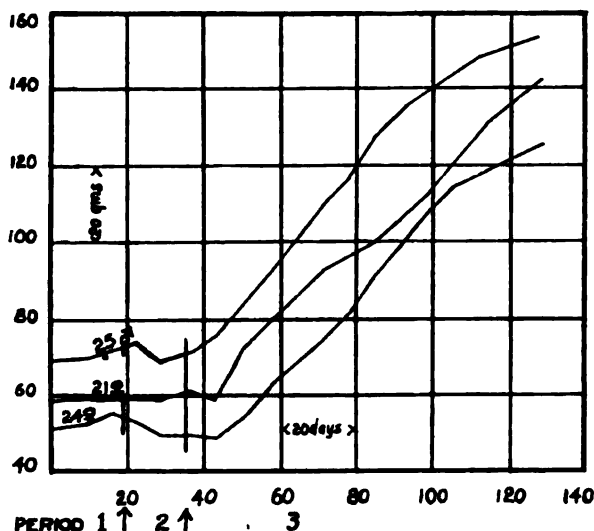


CHART 3. The curves illustrate the effect of incorporating the Mitchell and Nelson protein-free milk in place of the Osborne and Mendel product—the former being lower in protein nitrogen. Throughout, this protein-free milk was present. In Period 1 (maintenance) there was 7 per cent corn gluten protein; in Period 2, 6.5 per cent protein of which 22 per cent was lactalbumin and 78 per cent corn gluten; and in Period 3, 8 per cent protein of which 36 was lactalbumin and 64 per cent corn gluten. It will be seen that good growth took place in Period 3, when the lactalbumin supplemented the corn gluten to the extent of 36 per cent with the Mitchell and Nelson protein-free milk present.

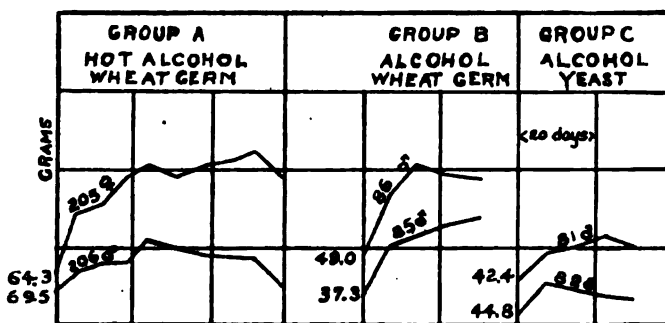


CHART 4. The curves illustrate the poor growth obtained with lactalbumin as the sole protein when protein-free milk was not used. The diet was made up of 10 per cent lactalbumin protein, 3.7 per cent salt mixture 185(McCollum), 18 per cent butter fat, 10 per cent lard, 5 per cent of the hot alcohol extract of ether-extracted wheat germ or 1 per cent of the cold alcohol extract of dried brewers' yeast, and starch to make up the balance.

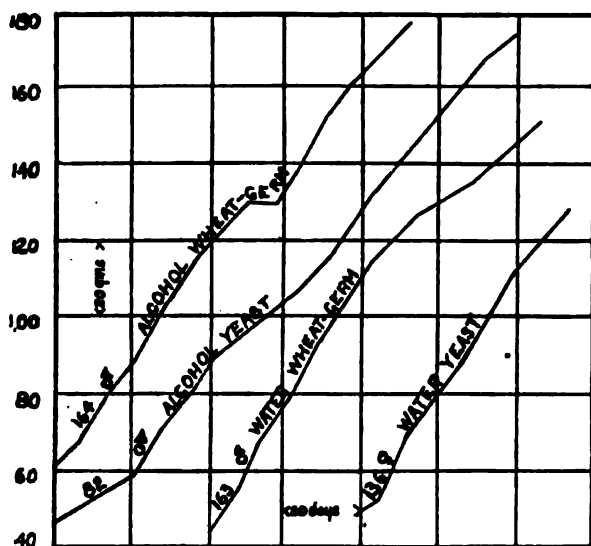


CHART 5. The rations fed these groups of rats were composed of 10 per cent lactalbumin protein, 18 per cent butter fat, 10 per cent lard, 3.7 per cent salt mixture 185 (McCollum), 24.6 per cent purified lactose, water and alcohol extracts of either wheat germ or yeast to supply the water-soluble B vitamine, and starch to complete the diet. Normal growth is possible with each of these four diets even in the absence of protein-free milk.

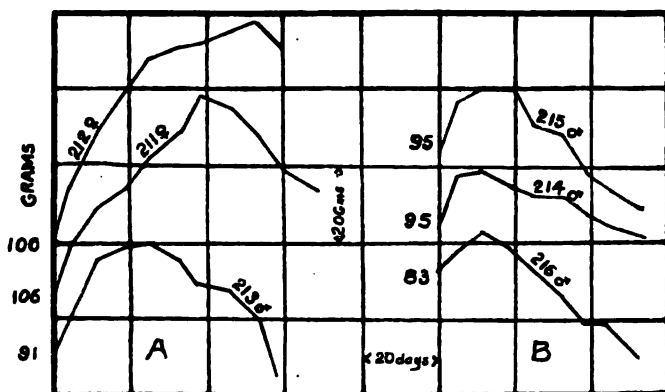


CHART 6. In these rations, the purified lactose was incorporated as the sole carrier of the water-soluble B vitamine. The diet for Group A was butter fat 5 per cent, lactalbumin protein 10 per cent, purified lactose 20 per cent, salt mixture 185 (McCollum) 3.7 per cent, and starch. The diet for Group B was the same as for Group A except that it contained less starch and more fat, butter fat 18 per cent and lard 10 per cent. The curves indicate that both the low and high fat diets are deficient—slight growth occurring at the start which is soon followed by a cessation of growth and subsequent decline.

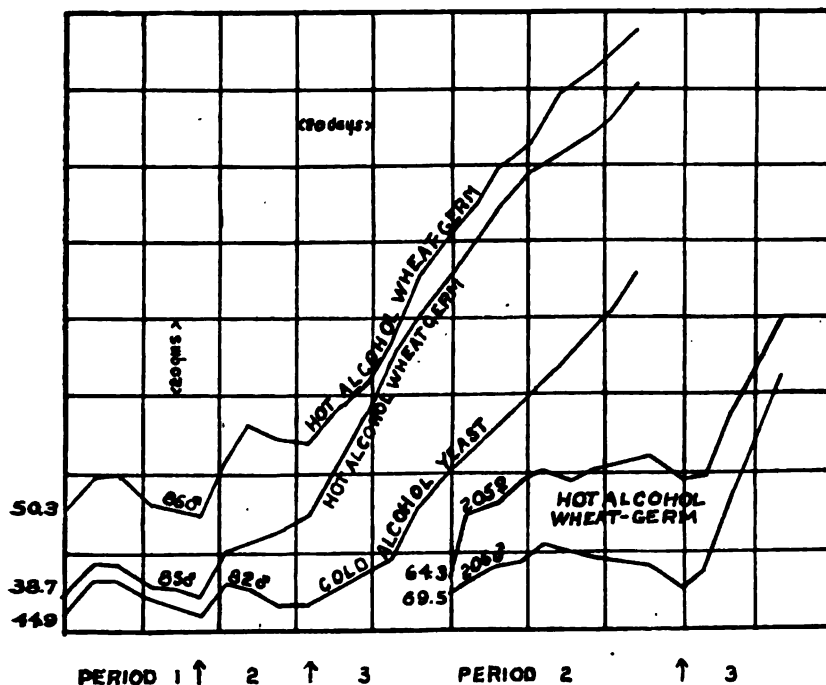


CHART 7. The curves show that lactose is an essential factor in the diet when lactalbumin is the sole protein. The basal ration throughout was lactalbumin protein 10 per cent, salt mixture 185 (McCollum) 3.7 per cent, butter fat 18 per cent, lard 10 per cent, and starch. The diet in Period 1 contained no water-soluble B accessory; in Period 2, this vitamin was supplied by an alcohol extract of wheat germ or yeast; in Period 3, 24.6 per cent of purified lactose replaced part of the starch of the ration used in Period 2. The growth response which followed the introduction of the purified lactose shows very definitely the importance of this constituent in these diets.

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RELATIVE ABUNDANCE OF SERUM PROTEINS IN ALBINO RATS AT DIFFERENT AGES.

By IKUZO TOYAMA.

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(Received for publication, March 6, 1919.)

Wells (1913) studied the relative abundance of serum proteins in rabbits as affected by age as well as by diet. Wells' observation is fundamentally important as the data not only reveal the influence of age, which is much neglected in such studies, but also form the basis for further experimental investigations. It appears desirable that the observation of Wells should be repeated or extended to other laboratory animals which are commonly used. I therefore have made a similar study on the serum of albino rats as these animals are extensively used and furthermore at The Wistar Institute rats from the colony of standard animals can be had for study.

The albino rats used in these experiments were fed on the ordinary laboratory diet and the blood was removed 24 hours after feeding. Suckling rats were isolated from the mother 8 to 16 hours before bleeding. The rats were etherized and bled directly from the carotid artery into a test-tube, care being taken not to sever the trachea or esophagus. The test-tube was closed to prevent evaporation and left at room temperature until the serum had separated. The blood was then centrifuged and the clear serum pipetted off and analyzed. In this work the method devised by Robertson (1915) was used; the total proteins were precipitated by 0.02 N acetic acid and the globulins by ammonium sulfate.

Altogether 66 specimens of serum, secured from 117 albino rats, were classified into fourteen groups according to age. In the majority of specimens, namely in 44, the desired amount of serum (1.5 to 2.0 cc.) was obtained from one rat, while in the younger rats, it was obtained by using from two to seventeen rats at once, the number increasing with decreasing age.

Refractive Index of Serum of Albino Rats at Different Ages.

Hatai (1918) found that the refractive index of the serum of albino rats varies clearly according to their age and in general increases with increasing age. Hatai noticed three distinct phases in the graph. The first phase is represented by the period of suckling, that is about the first 18 days, during which the refractive index rises rapidly. This period of rapid rise is followed by sudden fall of the graph, after which it rises again until the animal is about 70 to 90 days old. The end of this phase just precedes puberty. After 70 to 90 days of age the graph rises very slowly throughout the remainder of the span of life. The period of transition between the end of sexual maturity and the beginning of the adult phase is marked by irregularities in the graph, although no definite interpretation of this phenomenon has been given by Hatai.

For the rats employed in the present investigation I have also determined the refractive index of the serum. The results are given in Table I, Column 5, and also in the upper part of Chart 1.

Despite the fact of the small number (117) of rats examined by me, Chart 1 clearly shows in the upper graph the three phases mentioned by Hatai (1918). In my series, however, the end of both the suckling phase and the end of the puberty phase come several days later than in the records by Hatai; that is, at about 22 days for the former instead of 18 days, as shown by Hatai's rats, and for the latter at 85 instead of 70 days. This difference in the age at which the two critical phases occur is interesting because it is related to a period of poor nutrition in the rat colony in general, although the best animals obtainable were used for this work. It was noted at this time that the young were small for their age and required to be left with the mother longer than usual. The absolute value of the refractive index was also less than that found by Hatai.

Proteins of Blood Serum.

The results of the determinations of the proteins are shown in Table I, and the graphic representation is given in the lower part of Chart 1. A discussion of the changes in relative proportions of the serum proteins, shown in Chart 1, follows.

. During the suckling phase, or for about the first 23 days, the percentage of total proteins and also of both albumin and globulins shows an increase with age. Reiss (1909) has also reported a similar increase of protein content in the human serum in the suckling period—after the period of very high content of protein which occurs during the first days of life in man.

From the end of the suckling period (23 days) to the age of 30 days, the percentage of globulins falls rapidly and then rises again

TABLE I.

Changes in Refractive Index as well as in the Proportional Amount of Serum Proteins in Albino Rats at Different Ages.

Age.	No. of samples analyzed.	Body wt. (average).	Body length (average).	N_D	Total proteins.	Albumina.	Globulina.	Non-protein substances.	Relative amount of	
									Albumin	Globulin.
days		gm.	mm.		per cent	per cent	per cent	per cent.		
Newborn.	1	—	—	1.34101	2.8	1.7	1.1	1.6	60.7	39.3
7	1	10.8	—	1.34095	2.9	1.6	1.3	1.5	55.2	44.8
14	2	18.3	82	1.34245	3.9	2.4	1.5	1.2	61.5	38.5
22	2	26.5	97	1.34399	4.4	2.3	2.1	1.6	52.3	47.7
30	5	24.7	96	1.34350	4.7	3.5	1.2	1.5	74.5	25.5
50	5	51.1	115	1.34508	5.0	2.8	2.2	1.5	56.0	44.0
80	12	80.8	144	1.34648	5.7	3.3	2.4	1.5	57.9	42.1
87	5	69.4	142	1.34694	6.2	4.0	2.2	1.3	64.5	35.5
94	7	76.8	146	1.34647	5.9	3.7	2.2	1.4	62.7	37.3
103	9	78.5	142	1.34745	6.2	3.8	2.4	1.5	61.3	38.7
140	4	130.8	171	1.34722	5.9	3.1	2.8	1.5	52.5	47.5
180	3	129.0	172	1.34700	6.3	4.2	2.1	1.3	66.7	33.3
305	6	177.9	191	1.34876	6.5	2.9	3.6	1.7	44.6	55.4
385	4	197.8	196	1.34699	6.0	2.7	3.3	1.4	45.0	55.0

until 50 days. The percentage of albumins, however, shows at first an increase and then a decrease, in opposition to the concentration of globulins. During this period of alteration in the proportion of globulin to albumin in the serum, the value for total proteins of the serum does not show any corresponding fluctuation, but is rising continuously. It is known that young rats do not take any large quantity of milk after 23 days of age, but probably solid food only. Wells (1913) found that milk-fed animals show

an increase in the relative amount of the globulins over the control series fed on a mixed diet. The alteration shown in the period between 22 and 30 days may therefore be due to the fact that the rat becomes accustomed to solid food so that the composition of the blood is slowly restored, and the ratios which the various proteins bore to the total protein before the change in diet reappear.

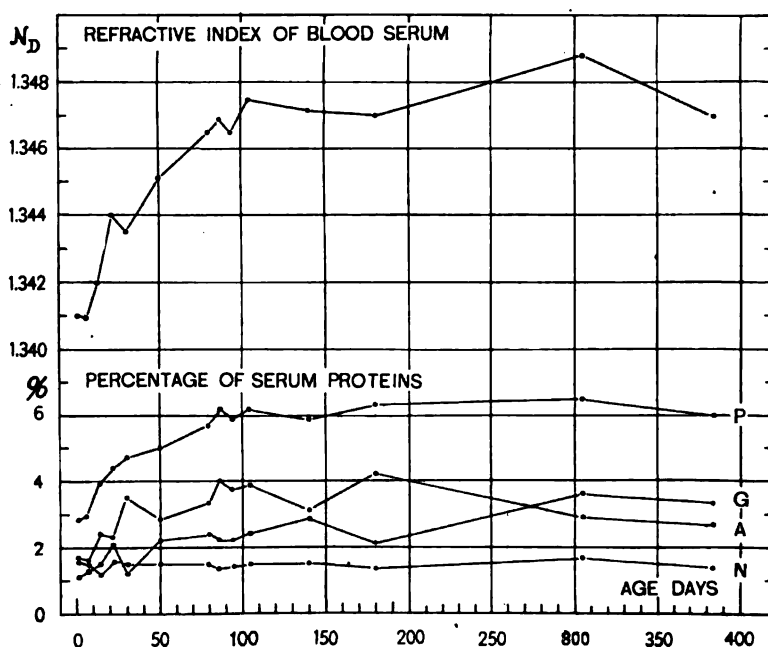


CHART 1. Changes in the refractive index and in the relative amount of serum proteins, based on the data given in Table I.

P = protein.

G = globulin.

A = albumin.

N = non-protein.

After adjustment to the change in diet the percentage of globulins and the percentage of albumins show a gradual increase with age from 50 to about 90 days. From puberty on the subsequent alteration in the percentage of total proteins is small, though it continues to rise throughout the rest of life. Between 80 to 180 days of age the relative proportion of globulins to albumins shows some irregularity, probably due to high individual variations.

Despite the fact of the irregularities just mentioned there is strong indication that the relative amount of albumin gradually decreases, while that of globulin gradually increases throughout the rest of life so far as examined. We thus find that at the age of about 275 days the ratio of globulin to albumin becomes the reverse of that found at an earlier age.

It should be stated that we always made careful postmortem examinations, and the blood of only those animals which were free from lung infection, as well as any other noticeable pathological alterations, was used for the purpose of analysis.

We infer therefore that the changes in the relative proportion of globulin and of albumin noted in the older rats were not produced by the immediate pathological alterations nor any disease present at the time of killing. It is, however, conceivable that the animals might during their earlier life have suffered from infection, yet so long as we have no direct evidence that the older rats suffered from infectious diseases, I feel justified in making the tentative conclusion that the globulin fraction relative to the albumin fraction shows some increase with advancing age. The percentage of non-protein substances (N) shows no variation corresponding with the age of the rat.

From his observations on rabbits Wells (1913) concludes that the percentage of total proteins increases with age between 21 to 140 days, but that fully adult animals have a slightly lower content.

My observations on the rat, however, show no such tendency of decrease in the content of proteins, even with rats which are 300 days old, although the rats at 385 days do show a slight falling off. Hatai (1918) questions whether or not the adult rabbits used by Wells were entirely normal, and so far as my own data are concerned the rats which were free from visible disease show no indication of falling off, when fully adult and even older. On the contrary, there is a strong tendency for steady increase of the proteins. Wells' data show no decisive increase of the relative amount of globulins at even 1 year of age, while the rats 300 days old give a greater relative amount of globulin than of albumin, thus reversing the relation present at younger periods. I am unable to state whether the serum of rabbits older than 1 year will show similar alterations in the serum proteins as are noted here in the rat.

CONCLUSIONS.

1. The present observation supports the view held by Hatai (1918) that there are in the albino rats three distinct phases in the growth curve of the refractive index of serum; namely, suckling, puberty, and maturity.

2. The percentage of total proteins increases very rapidly during the suckling period, but during the puberty phase growth is slow. The percentage of proteins during the adult period shows only slight increase, but at the age of 385 days a small fall was noticed; this may or may not be significant.

3. The percentage of albumin, though irregular, shows rapid increase for the first 30 days of age. This increase is followed by a rapid fall which soon is followed by a steady rise until the end of sexual maturity. The albumin content at the adult period shows first slow and later a steady relative diminution. The globulin fraction also shows a rapid increase until the end of the suckling period. This rise is followed by a sharp fall and reaches a minimum at the age of 30 days, caused probably by the change of diet from milk to solid food. The relative amount of globulin then shows a steady increase, and at the age of about 275 days the relative amount of globulins becomes greater than that of the albumin.

4. The percentage of the non-protein bodies is practically constant throughout the entire span of life.

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STUDIES OF ACIDOSIS.

XIII. A METHOD FOR TITRATING THE BICARBONATE CONTENT OF THE PLASMA.

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(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, April 1, 1919.)

For determination of carbonates and bicarbonates in the absence of proteins and other buffers methods of two general types are available: (1) measurement of the carbon dioxide evolved when the carbonates are decomposed with acid, and (2) titration of the basic part of the carbonate molecule, either by adding excess acid, boiling off the CO_2 , and titrating back, or by using an indicator which, like methyl orange, is not sensitive to the weak acidity of carbonic acid. Hitherto only methods of the first type, based on carbon dioxide determination, have proven accurate and practicable for blood analysis.

In order to titrate accurately the bicarbonate of the blood plasma, it is both theoretically and actually necessary, as a net result of the operation, to transform the bicarbonate into the salt of whatever acid is used in the titration, without altering the normal hydrogen ion concentration of the plasma. If the hydrogen ion concentration is altered, the proteins of the plasma bind as a result either acid or alkali in amounts different from those which they bind *in vivo*. Consequently, under such conditions the proteins act either to increase the acid added in titration to an amount more than equivalent to the bicarbonate, or to depress it below the bicarbonate equivalent.

So far as we are aware, no method for titration of the blood plasma with indicators has yet been proposed which meets the following three requirements, all of which are necessary in order to permit interpretation of the results in terms of plasma bicarbonate: (1) The use of the hydrogen ion concentration of nor-

mal blood as the end-point; (2) Removal of the carbon dioxide set free by the added acid; (3) Use of an indicator not affected by the plasma proteins.

It appeared probable that if these requirements could be met, a simple titration of the plasma bicarbonate might be possible, and recent investigations have provided the theoretically necessary data. The work of Lundsgaard (1912), who first demonstrated the constancy of the hydrogen ion concentration of the blood, of Hasselbalch (1911, 1913, 1915), and of a number of subsequent investigators (Michaelis, 1914; Peabody, 1914; Milroy, 1914; Parsons, 1917; Sonne and Jarløv, 1918), has shown that the pH of human blood under nearly all conditions, normal and pathological, even in acidosis, is approximately constant at 7.4. McClendon has shown that when excess acid is added to the blood plasma, the CO_2 driven off by a stream of hydrogen, and alkali added until the normal pH, as determined by the gas chain, is restored, the acid consumption is about what would be expected from the average bicarbonate content established by Van Slyke, Stillman, and Cullen (1917). An indicator capable of showing pH 7.4 accurately, in the presence of the plasma proteins, has been found in neutral red by Miss Annie Homer (1917). The elimination of carbon dioxide is merely a matter of efficient aeration. Consequently the difficulties to the titration appear to have become theoretically surmountable, and we have found in fact that the simple titration technique described below gives results for plasma bicarbonate identical with those yielded by determining the carbon dioxide.

Description of Method.

In drawing and centrifugating the blood the precautions outlined by Van Slyke and Cullen (1917), for preventing loss or accumulation of carbon dioxide and consequent change in the distribution of bicarbonate between corpuscles and plasma, are to be observed. Oxalate plasma is used. Up to the beginning of the analysis, the blood and plasma are handled exactly as described for the carbon dioxide method.

For the analysis 2 cc. of plasma are pipetted into a round-bottomed flask of 150 to 200 cc. capacity, and 5 cc. of 0.02 N hydrochloric acid are added (this is about 2 cc. of 0.02 N acid in

excess of the bicarbonate normally present). In order to remove the carbon dioxide set free by the acid, the flask is shaken vigorously with a rotary motion, so that the solution is whirled in a thin layer about the inner wall. 1 minute of this treatment is sufficient to remove the carbonic acid so completely that not enough is left to affect the results measurably. The solution is now poured as completely as possible into a 50 cc. Erlenmeyer flask and the walls of the larger flask are rinsed with 20 cc. of water. The water is measured within 1 cc. in a cylinder, and approximately a third is used for each of three washings.

When the solution, measuring about 26 cc., has been transferred to the 50 cc. Erlenmeyer flask, 0.3 cc. of a 0.1 per cent solution of neutral red (dissolved in 50 per cent alcohol) is added. 0.02 *N* carbonate-free NaOH is then run in from a burette (preferably but not necessarily a "micro-burette") until the color of the solution matches that of 29 cc. of a standard phosphate solution, of pH 7.4, contained in a similar 50 cc. flask.¹

In place of neutral red, 0.3 cc. of a 0.04 per cent solution of phenolsulfonephthalein may be used as indicator, and gives an

¹ The standard solutions of pH 7.2 and 7.4 may be made as follows (Clark and Lubs, 1917), M/20 solutions being obtained. The M/5 KH_2PO_4 contains 27.23 gm. of KH_2PO_4 per liter.

pH	M/5 KH_2PO_4	N/5 NaOH	
	cc.	cc.	
7.2	50	35.0	Dilute to 200 cc.
7.4	50	39.5	" " 200 "

They may also be made by Sørensen's method (Sørensen, 1912) from KH_2PO_4 and Na_2HPO_4 as follows, the phosphate concentration being M/15. Both salts prepared especially for this purpose may now be obtained from Merck and Co.

pH	Na_2HPO_4	KH_2PO_4	
	gm.	gm.	
7.2	6.89	2.47	Dilute to 1 liter.
7.4	7.72	1.67	" " 1 "

If crystalline $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ is used instead of the anhydrous Na_2HPO_4 , the amounts are increased to 8.66 and 9.67 gm. for the solutions of pH 7.2 and 7.4 respectively.

The plasma solution becomes somewhat turbid, but the turbidity partially clears up as the end-point is approached, and the latter can be determined within one drop of the 0.02 *N* NaOH.

end-point slightly more easy to distinguish than that of neutral red. When phenolsulfonephthalein is used, however, the standard solution must be of pH 7.2 instead of 7.4. The reason for this is that, as pointed out by Miss Homer (1917), when phenolsulfonephthalein is added to a solution containing serum proteins, the pH is considerably higher than that estimated by colorimetric comparison with standard phosphate solutions. The indicator suffers from a protein error. Under the conditions of the analysis here described, however, the error is quite constant at 0.2 to 0.3 pH; *i.e.*, if the color matches that of a standard solution of pH 7.4, the actual pH in the solution under examination is about 7.7 (see Table II). If a standard solution of pH 7.2 is used, however, the titration runs to the same point obtained with neutral red, and, as stated above, the end-point is, to most eyes, somewhat sharper than with neutral red.

For the titrations we have used a 3 cc. micro-burette of the model devised for use with the blood sugar method of Bang. It was provided with an automatic filling device and divided into 0.02 cc. divisions which were over 1 mm. apart, so that readings accurate to within 0.01 cc. could be made easily. The tip was drawn out to a fine point, so that the drops were small, measuring only 0.03 cc. each. Such a burette is extremely desirable, but not absolutely necessary. Even an ordinary 50 cc. burette divided into 0.1 cc. divisions may be used, an error of 0.1 cc. altering the result by only one-thirtieth of the normal, since the bicarbonate in 2 cc. of plasma normally neutralizes about 3 cc. of 0.02 N acid. The accuracy of the method, however, justifies the use of a finer burette, for our results seldom varied from the calculated values, or from agreement with duplicates, by more than 0.05 cc. and were usually within one drop, or 0.03 cc.

The End-Point.—With both indicators, a peculiar phenomenon occurs as the end-point is approached. Each drop appears to change the color past the end-point, but within a few seconds the color shifts back, and it is seen that at least another drop is needed before the genuine end-point is reached. Consequently, the final color comparison should not be made until at least 30 seconds after the last drop of 0.02 N NaOH has been added. Because of this behavior, as well as the character of the color change, it is well, particularly with neutral red, to overrun the end-point by a drop, rather than stop short of it when in doubt.

Calculation of Results.—The number of cc. of 0.02 N NaOH used in the titration is subtracted from the number required to neutralize to the same indicator 5 cc. of the 0.02 N HCl used. This number is, of course, approximately 5, but it usually varies slightly from that because of difference in the factors of acid and alkali, and because of the calibration error of the 5 cc. pipette used for measuring the acid. Consequently the maximum accuracy is obtained by performing a preliminary titration on 5 cc. of the acid plus 20 cc. of the distilled water, using the same pipette, indicator, and end-point as in the plasma titration.

The following is a typical calculation on a normal human plasma.

0.02 N NaOH = HCl added.....	5.09 cc.
0.02 N NaOH taken in titration.....	2.03 "
0.02 M NaHCO ₃ in 2 cc. plasma or	} 3.06 "
0.01 M NaHCO ₃ in 1 cc. plasma	
3.06 ÷ 100 = 0.0306 = molecular concentration of NaHCO ₃ in plasma.	
3.06 × 22.4 = 68.5 volume per cent CO ₂ bound as bicarbonate in the plasma.	

Since the titration result represents cc. of 0.01 M NaHCO₃ per cc. of plasma, it is transformed into terms of molecular concentration of NaHCO₃ in the plasma merely by dividing by 100.

For the sake of comparison with results of bicarbonate determination by the CO₂ method (Van Slyke and Cullen), the molecular concentration is multiplied by 2,240, in order to give results in terms of cc. of CO₂ per 100 cc. of plasma. According to the gas laws the amount of CO₂ contained in a M carbonate solution is 22,400 cc. per liter (measured as CO₂ gas at 0°, 760 mm.) or 2,240 cc. of gas per 100 cc. of solution. Hence multiplying the bicarbonate molecular concentration by 2,240, or multiplying the cc. of 0.02 N acid used in the titration by 22.4, gives the volume per cent of bicarbonate CO₂ in the plasma.

Inversely, of course, dividing the volume per cent of CO₂, as determined by Van Slyke and Cullen, by 2,240 transforms the CO₂ figures into terms of molecular concentration.

The Standard 0.02 N Sodium Hydroxide.—The 0.02 N NaOH as a basis for the determination must, in order to maintain its value, be protected from contact with atmospheric carbon dioxide and from glass. Even standing over night in a burette of soft glass is

likely to result in the solution of enough alkali to raise the titration value of the standard solution. The standard alkali should be kept in paraffined bottles, and the burette filled with fresh solution each day that it is used.

In order to obtain a carbonate-free alkali solution, we use the well known expedient of first dissolving the NaOH in an equal weight of water. Sodium carbonate is insoluble in such a concentrated alkali solution and settles to the bottom. 5.5 cc. of the clear supernatant solution diluted to 5 liters yields an approximately 0.02 N solution which is standardized by titration with neutral red against 0.02 N HCl. In performing the titration it is preferable to run the acid into the alkali, thus titrating from the yellow alkaline color to the acid red. The color change in this direction occurs without the time lag observed when alkali is added to acid.

EXPERIMENTAL.

Determinations on Known Carbonate Solutions.—Solutions of Merck's c.p. Na_2CO_3 were made in concentrations ranging from the carbonate content of normal plasma downwards, and titrated as described for plasma. The results are given in Table I.

Accuracy of End-Point in Plasma Titrations.—Table II shows the results of a number of titrations. After the titrations were finished the pH values of the solution were checked by means of the gas chain. It is seen that the desired value of pH 7.4 was closely approximated as the end-point in each titration, when neutral red was used as indicator with a standard of pH 7.4 for comparison, or when phenolsulfonephthalein was used as indicator with a standard of pH 7.2 for comparison. With the latter indicator, however, and a color standard of pH 7.4, the titration, due to the protein error pointed out by Miss Homer (1917), went to a pH of 7.7. Even this change altered the results by only about 0.001 M, and when a color standard of pH 7.2 was used with phenolsulfonephthalein, the error was completely corrected.

Time Required for Removal of Carbon Dioxide by Aeration.—A series of determinations was performed with portions of the same horse serum, exactly as outlined above under "Description of Method," except that the aeration period was varied. The results in Table III show that an aeration period of 1 minute is

TABLE I.

Na ₂ CO ₃ per liter by weight.*	Concentration of carbonate by methyl orange titration.		Concentration of carbonate by neu- tral red titration with technique used for blood plasma.	
	0.02 N HCl = 20 cc. carbonate solution.	Concentration calculated as NaHCO ₃ .	0.02 N HCl = 2 cc. solution.	Concentration calculated as NaHCO ₃ .
gm.	cc.	molecular	cc.	molecular
1.590	29.1	0.0291	2.90	0.0290
1.090	19.7	0.0197	1.95	0.0195
0.727	13.1	0.0131	1.29	0.0129
0.363	6.57	0.0066	0.67	0.0067

* The carbonate was not dried, hence the actual concentration indicated by the methyl orange titration is only 97 to 98 per cent that indicated by the weight of carbonate taken.

TABLE II.

Serum.	0.02 N NaOH = HCl taken.	0.05 N NaOH used in back titration.	0.02 N NaOH = NaHCO ₃ in 2 cc. plasma.	Concentration of NaHCO ₃ .	Indicator used.	pH of standard phos- phate solution used for end-point com- parison.	pH of plasma solu- tion at end of titra- tion.
	cc.	cc.	cc.	molec- ular			
Horse 1.	5.13	2.41	2.72	0.0272	Neutral red.	7.4	7.36
	5.13	2.44	2.69	0.0269	" "	7.4	7.41
	5.12	2.56	2.56	0.0256	Phenolsulfonephthalein.	7.4	7.72
	5.12	2.53	2.59	0.0259	"	7.4	7.71
	5.12	2.43	2.69	0.0269	"	7.2	7.45
Horse 2.	5.35	2.60	2.75	0.0275	Neutral red.	7.4	7.43
	5.35	2.63	2.72	0.0272	Phenolsulfonephthalein.	7.2	7.45

TABLE III.

Duration of aeration.	Bicarbonate concentration found by titration.
min.	molecular
0	0.0117
0.25	0.0220
0.5	0.0255
1.0	0.0260
2.0	0.0260
3.0	0.0260

sufficient, but that 30 seconds is probably not quite enough. Incidentally the result of the titration performed without aeration shows the impossibility of a correct titration without removal of carbon dioxide.

Parallel Determinations of Plasma Bicarbonate by Titration and by Carbon Dioxide Capacity.—The results in Table IV were obtained on the plasmas of blood samples drawn from the ear veins of rabbits and the arm veins of men. The carbon dioxide capacities were determined by one of us, while the titrations were being performed by another.

Parallel Determinations by Titration and Carbon Dioxide Capacity Methods on Normal Human Plasma plus Known Amounts of Acetic

TABLE IV.

Plasma.	CO ₂ bound as bicarbonate by 100 cc. of plasma:	Bicarbonate concentration calculated from CO ₂ ($\frac{\text{CO}_2}{2,240}$).	Bicarbonate concentration determined by titration.
	cc.	molecular	molecular
Normal Rabbit 61.....	56.7	0.0253	0.0270
“ “ 62.....	66.9	0.0298	0.0305
“ “ 63.....	46.2	0.0206	0.0199
Diabetic. No acidosis.....	58.2	0.0259	0.0240
Normal man.....	61.6	0.0275	0.0265

Acid.—Acetic acid has practically the same dissociation constant (1.8×10^{-5}) as β -hydroxybutyric (2.0×10^{-5}), and the effect on acid-base balance produced by adding it to plasma is therefore practically identical with the effect of β -hydroxybutyric acid.

The blood was drawn from one of the authors. 5 cc. portions were treated with water, or water plus 0.1 N acetic acid, making the total volume up to 6.5 cc. Portions were then taken for titration and for carbon dioxide capacity determination (Van Slyke and Cullen method), as described in the preceding paragraph. Table V gives the results. The CO₂ results are reduced to terms of molecular concentration by dividing the volume per cent CO₂ by 2,240, the theoretical gas constant, as described on page 171. The results are plotted in Fig. 1.

*Discussion of the Titration and Carbon Dioxide Capacity Methods
for Determination of Plasma Bicarbonate.*

It is evident from Fig. 1 that the titration gives theoretically correct values over the entire range of bicarbonate concentrations from normal to zero. The CO_2 determinations agree both with the titration results and with the calculated values for a range of the latter from normal down to somewhat below 0.01 M bicarbonate (22.4 per cent CO_2 capacity). When the bicarbonate falls as low as 0.003 M, however, (6.7 per cent CO_2 , or about one-tenth the normal), the CO_2 method gives higher bicar-

TABLE V.

Plasma.	0.1 N acetic acid.	Water.	CO ₂ bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in concentration.		Concentration of acetic acid added.
				By CO ₂ capacity.	By titration.	By CO ₂ capacity.	By titration.	
cc.	cc.	cc.	cc.	molecular	molecular	molecular	molecular	molecular
5	0	1.5	58.9	0.0267	0.0264	0	0	0
5	0.478	1.0	41.6	0.0186	0.0192	0.0081	0.0072	0.0073
5	1.036	0.5	24.2	0.0108	0.0106	0.0155	0.0158	0.0159
5	1.514	0.0	12.8	0.0057	0.0028	0.0205	0.0236	0.0233
5	1.036 of 0.2 N	0.5	0.0	0.0000	-0.0050	—	0.0314	0.0319

bonate than is actually present. This fact was demonstrated and discussed in the original paper on the carbon dioxide capacity method (Van Slyke and Cullen, pp. 327, 328) in the following words:

"Until acid equivalent to about half the plasma bicarbonate has been added the fall in bicarbonate approximately equals in molecular equivalents the amount of acid added. As the amount of acid becomes greater, however, the drop in plasma bicarbonate begins to fall short of the added acid. This is due to the fact that the H_2CO_3 concentration is kept constant, instead of being reduced in proportion to the bicarbonate. The condition is similar to that of the blood in uncompensated acidosis. The $\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}$ ratio, and consequently the C_H is increased. As a result the other plasma buffers (chiefly proteins) bind a measurably greater amount of acid than they could at normal C_H , and the acid so bound is prevented from decomposing bicarbonate.

"The effect on the routine plasma determination is that the bicarbonate determined by our $[\text{CO}_2]$ technique denotes a fall in the more severe stages of acidosis which is not quite so great as the actual drop in bicarbonate *in vivo*. The relationship between added acid and decrease in bicarbonate, however, is made so constant by saturating the plasma at a definite carbon dioxide tension that the lack of absolute numerical proportionality in the lower ranges is no practical detriment to the interpretation of results."

In some types of experimental work the theoretical perfection of the titration will doubtless give it an advantage, but for ordi-

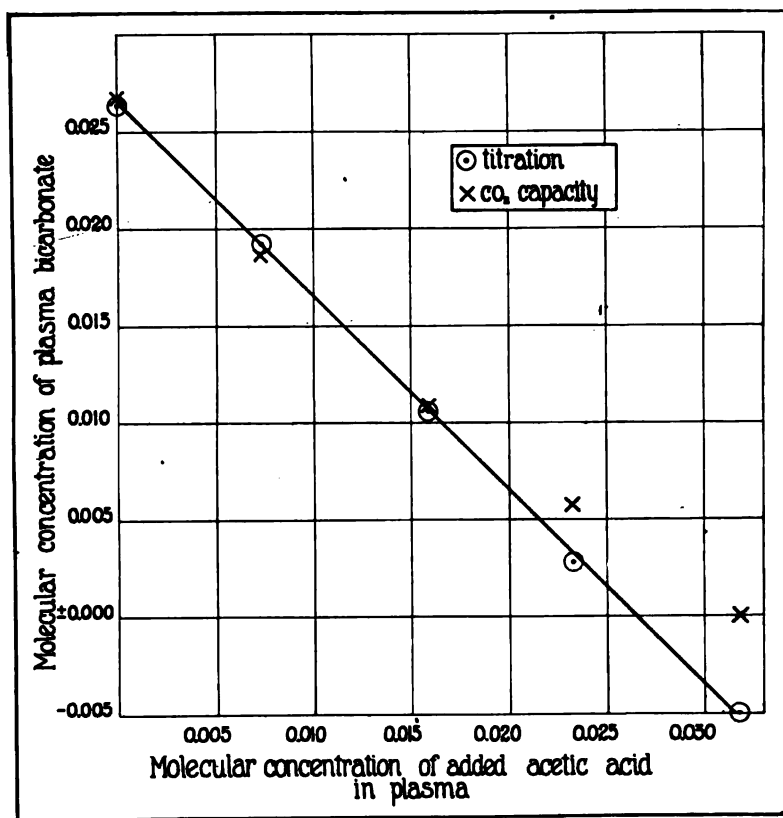


FIG. 1. Effect of addition of acetic acid on bicarbonate concentration of normal human plasma. The straight line represents the bicarbonate concentration calculated on the assumption that the fall in bicarbonate is equivalent to the amount of acid added to the plasma. The results of titrations are represented by \circ , the results of CO_2 determinations by \times .

nary purposes, especially for clinical diagnosis, the choice between the two methods appears entirely one of convenience. The carbon dioxide capacity has the advantage that the extraction and measurement of the gas require slightly less time than even the few minutes necessary for a titration, and require no accurate standard solutions; while the titration has the advantage that it requires no special apparatus. Within the bicarbonate range ordinarily encountered even in acidosis, (Stillman, Van Slyke, Cullen, and Fitz, 1917), the results of either method apparently may be interpreted in terms of the other, so closely do the two agree, and each method is capable of accuracy to within 1 or 2 per cent of the amount of bicarbonate normally present.

CONCLUSION.

The bicarbonate content of serum or oxalate plasma is determined by adding an excess of standard acid (5 cc. of 0.02 N HCl to 2 cc. of plasma), removing the carbon dioxide by rotating the solution for 1 minute about the wall of a flask, and titrating back with 0.02 N NaOH to the original hydrogen ion concentration of blood (pH 7.4) with neutral red as indicator.

The results agree closely with those of the carbon dioxide capacity method over the range of bicarbonate concentrations (0.03 to 0.01 M) ordinarily encountered in man, even in severe acidosis. Below this range the titration continues to give accurate results, while the CO₂ capacity method gives, for reasons discussed in the original paper on the CO₂ method, somewhat higher values. For clinical and most experimental purposes, however, it appears that the two methods give so nearly identical results that they may be used interchangeably.

Micro-titration.—When it is desirable to employ only small amounts of plasma, 0.400 cc. may be transferred to a test-tube of about 20 mm. diameter, and 1.000 cc. of 0.02 N HCl added. The tube is laid in a nearly horizontal position, so that the layer of liquid extends for about 10 cm. along the lower side. The tube is then rotated or rolled back and forth vigorously, but not so roughly as to cause foaming, for not less than 1.5 minutes, in order to cause the carbon dioxide to escape. Three drops of indicator are then added, and the solution is titrated in the test-tube with 0.004 N NaOH to a pH of 7.4, using as a color standard a like volume of phosphate solution in a similar tube.

The 0.004 N NaOH is so rapidly altered by contact with either glass or atmospheric carbon dioxide that it is advisable to make it fresh for each series of analyses by diluting 10 cc. of 0.1 N NaOH to 250 cc. with distilled water that has been freed of CO₂ either by boiling or by shaking in an evacuated flask. The control titration of the 0.004 N NaOH against the 1 cc. of 0.02 N HCl should be performed immediately before the plasma titration.

The calculation is the same as in the larger titration. The number of cc. of 0.004 N NaOH used is subtracted from the number of cc. (approximately 5) required to neutralize the HCl in the control titration. The difference divided by 100 represents the molecular concentration of bicarbonate in the plasma, while the difference multiplied by 22.4 indicates the volume per cent of bicarbonate CO₂.

With care in the calibration of pipettes, and especially in the control of the 0.004 N NaOH, results nearly and perhaps quite as accurate as in the larger titration appear attainable.

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE LIQUEFACTION OF GELATIN.

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Fischer and Coffman¹ show that "there is a progressive increase in the tendency of gelatin to go into solution in mixtures of the salts of polybasic acids as the amount of acid or alkali in these mixtures is increased from a given low point."² In view of the well known relation of hydrogen ion concentration to various equilibria, it seemed desirable to investigate its effect in the liquefaction of gelatin since the results of Fischer and Coffman do not cover this point directly. Also it seemed desirable that the results of Fischer and Coffman be checked, as they have an interest wider than the physiological application. The present paper is intentionally limited to a presentation of chemical and physical data, and does not discuss their medical applications.

According to the scheme of Fischer and Coffman, solutions were made up from known volumes of standardized acids, salts, and alkalies so that a graded variation from acid to alkali through neutral solution was secured, gelatin solution of definite strength being added in each case and the whole made up to a given volume (10 cc.) with conductivity water. Thus the gelatin was always contained in the same volume of water. The pH values in gelatin solutions were determined by the customary method of adding a few drops of indicator solution and comparing the color just produced with the color of the same quantity of the same indicator in standard solutions of known hydrogen ion concentration (checked electrometrically). A blank test of each solution was

¹ Fischer, M. H., and Coffman, W. D., *J. Am. Chem. Soc.*, 1918, xl, 304.

² The isoelectric point for gelatin is given as pH 4.60 by Michaelis and Grineff, cited by Walpole, G. S., *Biochem. J.*, 1914, viii, 638.

made colorimetrically for the pH^* value in absence of gelatin; also, a test on the pH of the solution of gelatin alone in conductivity water.

Table I shows the composition and pH of phosphoric acid (approximately 0.01 N) through phosphates to sodium hydroxide in twenty-one stages, the higher concentration in alkali not being measured colorimetrically owing to lack of a suitable indicator, and for our present purpose being so far from the liquefaction pH of gelatin as not to warrant the extra work of electrometric measurement at this time. In Table I is also given the effect of the solutions cited upon the physical state of gelatin (3 per cent) at 21°C ., together with the pH of each gelatin-phosphate system as determined by indicators.⁴ The data in Table I are shown graphically in Fig. 1, where the number of the solution is indicated on the ordinate (from Nos. 2 to 21, passing from acid to alkali) and the observed pH, shown by the indicator, as on the abscissa. Curve 1 represents the solutions alone; Curve 2 shows the effect of the 3 per cent gelatin. The amphoteric effect of the gelatin is clearly in evidence, the hydrogen ion concentration being depressed in acid solution and raised in alkaline solution.

The gelatin itself in water showed acid reaction to the extent of $\text{pH} = 5.6$. The gelatin was the best obtainable for culture work, and was redissolved, filtered, and precipitated from 95 per cent alcohol twice, and dried at 105°C . for some hours. Between pH 8.4 and pH 9.2 the gelatin begins to liquefy and is not completely fluid at pH 9.6.

Table II shows the same effect of solutions upon gelatin using solutions approximately 0.1 N ranging from H_3PO_4 to NaOH . Fig. 2 is plotted in the same manner as Fig. 1, Curve 1 being again for the solutions alone, while Curve 2 shows the effect of adding 3 per cent of gelatin. In this series also the gelatin shows a marked

* $\text{pH} = \log \frac{1}{\text{concentration of hydrogen ions}}$ thus, a solution 0.01 normal with regard to hydrogen ions would have a $\text{pH} = 2$.

⁴ The authors are aware that a protein effect may be exercised upon the indicator by the gelatin, but this effect appears not to mask the pH determination for the present purposes. Most of the indicators are from the series of Clark and Lubs, (*J. Bacteriol.*, 1917, ii, 1) who chose them in part because of their relatively small errors in the presence of protein.

amphoteric effect, being acid in the alkali region and basic in the acid region.

This same amphoteric action is shown, using citric acid with 2 per cent gelatin and 3 per cent gelatin, in Tables III and IV and Fig. 3. In sodium bicarbonate solutions, Table V and Fig. 4, the gelatin always in alkaline solution displaces the pH toward the acid region. Thus, the action of gelatin in these buffer solutions tends to displace their pH value. The amphoteric action of gelatin in these solutions is a real effect.

From the data given in the tables, graphical representations of the effect of pH upon the liquefaction of gelatin have been secured by using an arbitrary ordinate scale of solidification; 100 being a set gel and 0 the mobile liquid, the semisolid condition is taken as 50. The abscissæ represent pH.

Fig. 5 shows that a 3 per cent gelatin liquefies between 8.4 and 9.2 in sodium bicarbonate solutions; between 8.4 and 10.1 in 0.01 N phosphoric acid solutions; and between 8.4 and 9.6 for citric acid solutions. The data are given in Tables I, IV, and V. Fig. 6, plotted from Tables III and IV shows the pH zone throughout which the gelatin remains solid in citrate solutions. As would be expected the stronger gelatin solution is solid over a wider pH range.

Fig. 7 shows the effect of temperature upon the setting of 3 per cent of gelatin in 0.1 N phosphoric acid-sodium hydroxide solutions from 18–25°C.; the narrowing of the pH range for solid gelatin solutions due to rise in temperature is clearly seen.

Throughout all this preliminary work the criterion of solidity in a set gelatin is rough, being simply that it must show a jelly-like elasticity.

CONCLUSIONS.

1. From the above data it is clear that the setting of gelatin is influenced by the hydrogen ion concentration of the medium and unless the gelatin is destroyed this effect is probably reversible.
2. Gelatin in the concentrations used is not without effect upon the buffer solutions, displacing the pH in such a manner as one would expect from an aggregate of amino-acids acting amphotERICALLY.

TABLE I.

Setting of Gelatin Solutions (3 Per Cent) with Phosphoric Acid through Phosphates to Sodium Hydroxide. Approximately 0.01 Normal Alkali.

Solution No.	Phosphate solution plus 5 cc. 6 per cent gelatin solution plus water to 10 cc. volume.							Phosphate solution plus water to 10 cc. without gelatin.		
	$\% \text{ H}_2\text{PO}_4$	$\% \text{ NaH}_2\text{PO}_4$	$\% \text{ NaHPO}_4$	$\% \text{ Na}_2\text{PO}_4$	$\% \text{ NaOH}$	pH	Indicator used.	Physical state 21°C.	pH	Indicator used.
	cc.	cc.	cc.	cc.	cc.					
1						5.6	M.R.	Solid.	7.2	P.R.
2	0.1					4.6	B.P.B.	"	2.3	T.B.
3	0.08	0.02				4.8	"	"	2.5	"
4	0.06	0.04				5.0	M.R.	"	3.2	B.P.B.
5	0.04	0.06				5.2	"	"	3.4	"
6	0.02	0.08				5.5	"	"	4.6	M.R.
7		0.10				5.7	"	"	6.0	B.C.P.
8		0.08	0.02			6.0	B.C.P.	"	6.7	"
9		0.06	0.04			6.3	"	"	7.0	P.R.
10		0.04	0.06			6.5	"	"	7.3	"
11		0.02	0.08			6.7	"	"	8.4+	"
12			0.10			7.1	P.R.	"	9.4	P.P.
13			0.08	0.02		7.5	"	"	10.0+	"
14			0.06	0.04		8.0	"	"		
15			0.04	0.06		8.4	"	"		
16			0.02	0.08		9.2	T.B.	Semisolid.		
17				0.10		9.6	"	"		
18				0.08	0.02	10. +	P.P.	Liquid.		
19				0.06	0.04			"		
20				0.04	0.06			"		
21				0.02	0.08			"		
22					0.10			"		

M.R. = methyl red.

B.P.B. = bromophenol blue.

B.C.P. = bromocresol purple.

P.R. = phenol red.

T.B. = thymol blue.

P.P. = phenolphthalein.

Setting of Gelatin Solutions (3 Per Cent) with Phosphoric Acid Through Phosphates to Sodium Hydroxide.

Solution No.	Phosphate solution plus 6 cc. 6 per cent gelatin solution plus water to 10 cc. volume.										Phosphate solution plus water to 10 cc. without gelatin.		
	N H ₂ PO ₄ cc.	N NaH ₂ PO ₄ cc.	N Na ₂ HPO ₄ cc.	N Na ₂ PO ₄ cc.	N NaOH cc.	pH	Indicator used.	Physical state.				pH	Indicator used.
								18°C.	20.5°C.	22°C.	25°C.		
1						5.2	M.R.	Solid.	Solid.	Solid.	7.2	P.R.	
2	1.0					2.8	M.Y.	Liquid.	Liquid.	Liquid.	1.3	T.B.	
3	0.8	0.2				3.2	B.P.B.	"	"	"	2.1	"	
4	0.6	0.4				3.6	"	Semisolid.	"	"	2.4	"	
5	0.4	0.6				4.0	"	Solid.	Semisolid.	"	2.8	"	
6	0.2	0.8				4.2	M.R.	"	"	Semisolid.	3.2	B.P.B.	
7	1.0					4.4	"	"	"	"	4.4	"	
8	0.8	0.2				5.+	"	"	Solid.	Solid.	5.8	B.C.P.	
9	0.6	0.4						"	"	"	6.4	"	
10	0.4	0.6						"	"	Semisolid.	6.8	"	
11	0.2	0.8				6.8	P.R.	"	"	"	7.2	P.R.	
12		1.0				7.3	"	"	Semisolid.	Liquid.	8.5	"	
13		0.8	0.2			7.9	"	"	"	"			
14		0.6	0.4					Semisolid.	"	"	9.6	T.B.	
15		0.4	0.6					Liquid.	"	"	10+	P.P.	
16		0.2	0.8					"	"	"			
17			1.0					"	"	"		not measurable with indicators at hand.	
18		0.8	0.2		0.2			"	"	"			
19		0.6	0.4		0.4	8.2	P.R.	"	"	"			
20		0.4	0.6		0.6	8.4	"	"	"	"			
21		0.2	0.8		0.8			"	"	"			
22			1.0		1.0	9.0	P.P.	"	"	"			

M.R. = methyl red.

M.Y. = metaniline yellow (*m*-benzolsulfonidiphenylamine).

B.P.B. = bromophenol blue.

P.R. = phenol red.

P.P. = phenolphthalein.

T.B. = thymol blue.

B.C.P. = bromocresol purple.

TABLE III.

Setting of Gelatin Solutions (2 Per Cent) with Citric Acid through Citrates to Sodium Hydroxide.

Citrate solution plus 5 cc. 4 per cent gelatin solution plus water to 10 cc. volume.									
Solution No.	N citric acid.	M NaH ₂ ci- trate.	M NaH ci- trate.	M Na ₂ ci- trate.	N NaOH	pH	Indicator used.	Physical state.	
								20°C.	12°C.
1	cc.	cc.	cc.	cc.	cc.	5.6	M.R.	Solid.	Solid.
2	1.0					4.3	B.P.B.	Liquid.	Liquid.
3	0.8	0.8				4.4	"	"	
4	0.6	1.6				4.4	"	"	
5	0.4	2.4					"	"	
6	0.2	3.2					"	"	
7		4.0					"	"	
8		3.2	0.8			5.0	"	"	
9		2.4	1.6				"	"	
10		1.6	2.4				"	"	Semisolid.
11		0.8	3.2				"	"	"
12			4.0				"	"	"
13			3.2	0.8			"	Semisolid.	Solid.
14			2.4	1.6		5.0	M.R.	"	"
15			1.6	2.4		5.2	"	Solid.	"
16			0.8	3.2		5.6	"	"	"
17				4.0		6.8	B.C.P.	"	"
18				3.2	0.2	9.0	P.R.	Liquid.*	Liquid.*
19				2.4	0.4	9.6	P.P.	"	
20				1.6	0.6	10. +	"	"	
21				0.8	0.8			"	
22					1.0			"	

* The system was turbid up to Solution 18 at both 20° and 12°, but at this point a clear liquid was obtained with some settled out precipitate. Evidently the gelatin was not of the highest grade.

M.R. = methyl red.

B.C.B. = bromocresol blue.

B.P.B. = bromophenol blue.

P.R. = phenol red.

P.P. = phenolphthalein.

TABLE IV.

Setting of Gelatin Solutions (3 Per Cent) with Citric Acid through Citrates to Sodium Hydroxide.

Solution No.	Citrate solution plus 5 cc. 6 per cent gelatin plus water to 10 cc. volume.						pH	Indicator used.	Physical state.
	$\frac{N}{4}$ citric acid.	$\frac{N}{4}$ NaH ₂ citrate.	$\frac{N}{4}$ NaH citrate.	$\frac{N}{4}$ Na citrate.	$\frac{N}{4}$ NaOH				
1	cc.	cc.	cc.	cc.	cc.		5.6	M.R.	Solid.
2	1.0						4.3	B.P.B.	" 2.3*
3	0.8	0.8					4.4	"	" 2.4
4	0.6	1.6					4.4	"	" 2.8
5	0.4	2.4					4.4	"	" 2.8
6	0.2	3.2					4.4	"	"
7		4.0					4.4	"	" 3.0
8		3.2	0.8				4.8	"	"
9		2.4	1.6				4.8	"	"
10		1.6	2.4				4.8	"	"
11		0.8	3.2				4.8	"	"
12			4.0				4.8	"	"
13			3.2	0.8			4.8	"	"
14			2.4	1.6			5.0	"	"
15			1.6	2.4			5.2	"	"
16			0.8	3.2			5.6	"	"
17				4.0			6.8	"	" 8.2
18				3.2	0.2		9.0	P.R.	Semisolid.
19				2.4	0.4		9.6	P.P.	Liquid.†
20				1.6	0.6		10. +	"	
21				0.8	0.8				
22					1.0				

* The numbers indicate pH values of the citrate solutions without gelatin.

† Very sharp change to mobile liquid.

M.R. = methyl red.

B.P.B. = bromophenol blue.

P.R. = phenol red.

P.P. = phenolphthalein.

TABLE V.

Setting of Gelatin Solutions (3 Per Cent) with Sodium Bicarbonate through Sodium Carbonate to Sodium Hydroxide.

Solution No.	5 cc. 6 per cent gelatin plus solutions tabulated plus water to 10 cc.							
	M NaHCO ₃ cc.	M Na ₂ CO ₃ cc.	M NaOH cc.	pH	Indicator used.	Physical state. 23°C.	NaHCO ₃ so- lution with- out gelatin pH.	Indicator used.
1				5.6	M.R.	Solid.		
2	0.20			7.5	P.R.	"	8.8	T.B.
3	0.18	0.02		8.4	"	"	9.3	"
4	0.16	0.04		8.6	"	Semisolid.	9.4	"
5	0.14	0.06		8.9	T.B.	"	9.5	"
6	0.12	0.08		9.2	"	Liquid.	9.6	"
7	0.10	0.10		9.4	"	"	10.0	P.P.
8	0.08	0.12		9.6	"	"		
9	0.06	0.14		10.0	P.P.	"		
10	0.04	0.16				"		
11	0.02	0.18				"		
12		0.20				"		
13		0.18	0.02			"		
14		0.16	0.04			"		
15		0.14	0.06			"		
16		0.12	0.08			"		
17		0.10	0.10			"		
18		0.08	0.12			"		
19		0.06	0.14			"		
20		0.04	0.16			"		
21		0.02	0.18			"		
22			0.20			"		

M.R. = methyl red.

P.R. = phenol red.

T.B. = thymol blue.

P.P. = phenolphthalein.

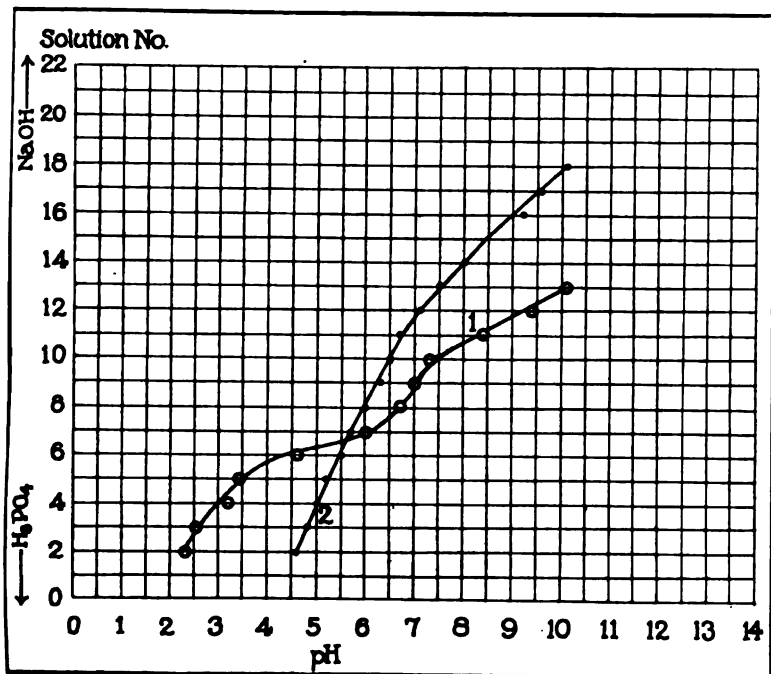


FIG. 1. Amphoteric effect of gelatin. Curve 1, pH of phosphoric acid 0.01 N through phosphates to sodium hydroxide; Curve 2, the same, but with 3 per cent gelatin.

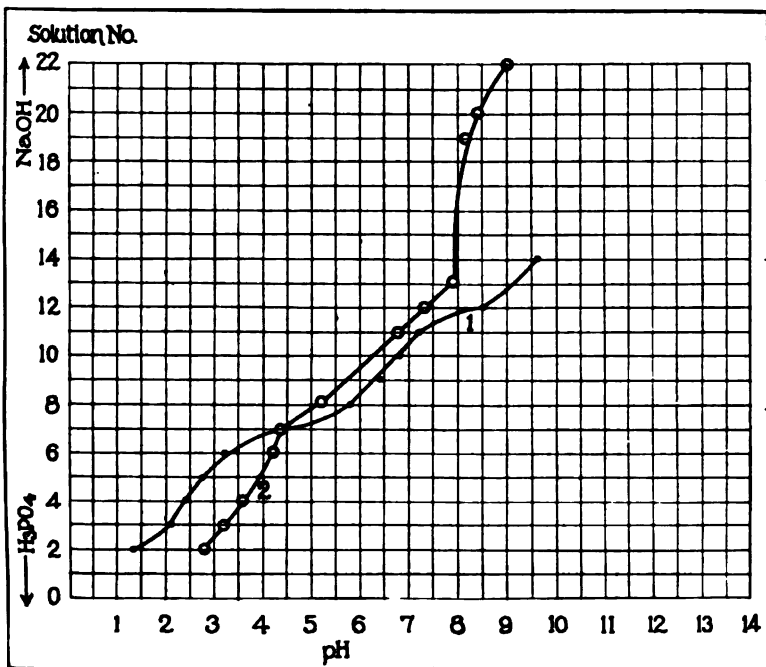


FIG. 2. Amphoteric effect of gelatin. Curve 1, pH of phosphoric acid 0.1 N through phosphates to sodium hydroxide; Curve 2, the same, with 3 per cent gelatin.

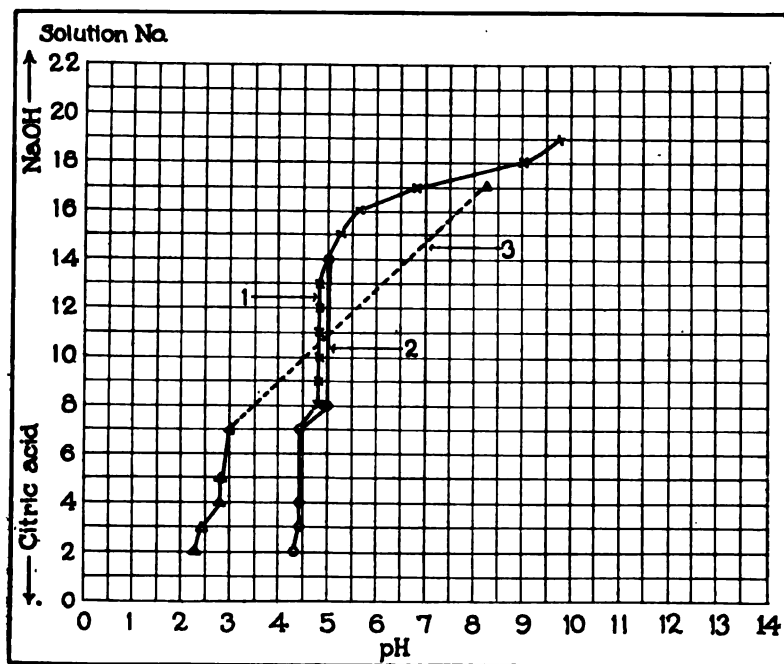


FIG. 3. Amphoteric effect of gelatin. Curve 1, pH of citric acid through citrates to sodium hydroxide with 2 per cent gelatin; Curve 2, the same, but with 3 per cent gelatin; Curve 3, the same, but with no gelatin present.

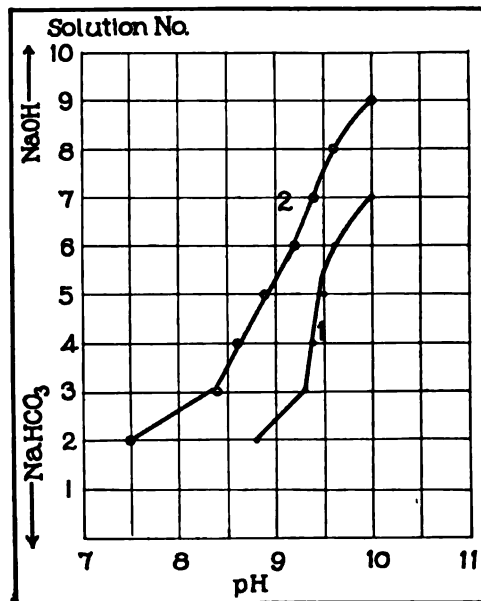


FIG. 4. Acid effect of gelatin. Curve 1, pH of sodium bicarbonate to sodium hydroxide; Curve 2, the same, with 3 per cent gelatin.

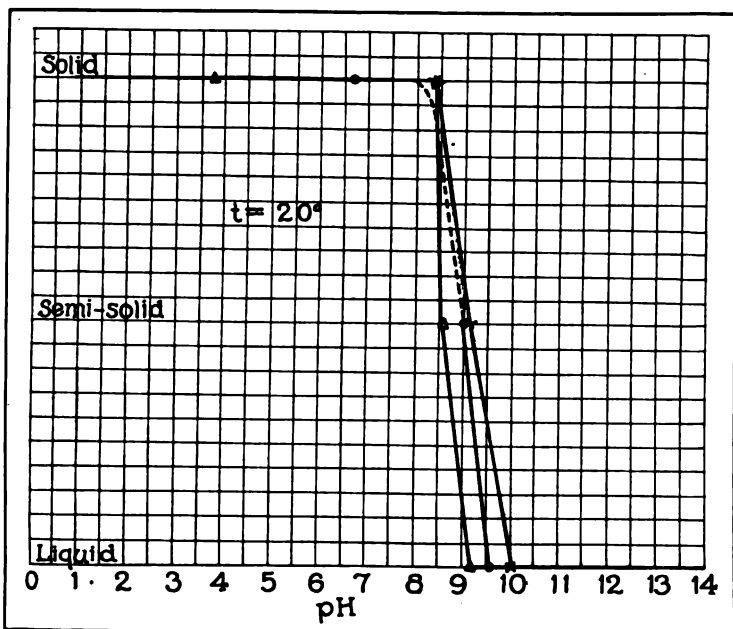


FIG. 5. Comparison of effect of citrate, phosphate, and bicarbonate solutions upon the setting of gelatin (3 per cent) at 20°C. O = citrates, X = phosphates, Δ = bicarbonates.

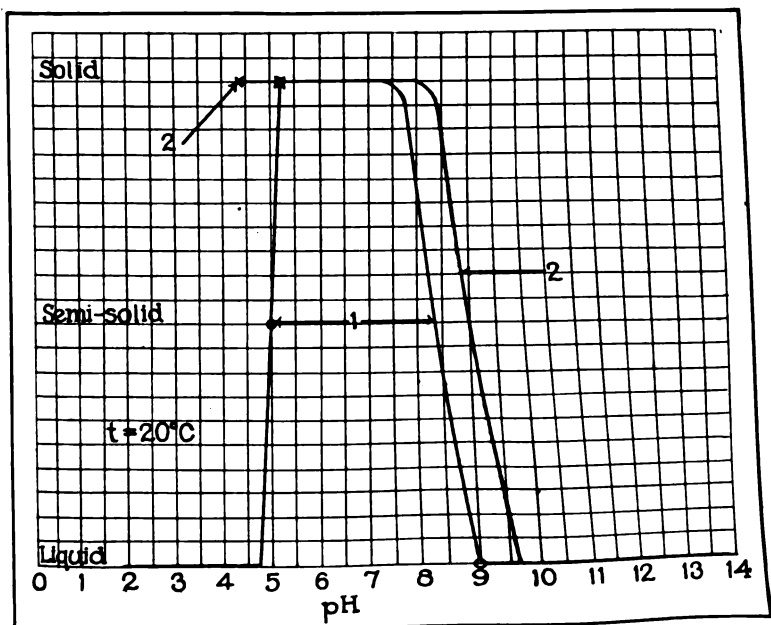


FIG. 6. Effect of pH upon setting of gelatin in citric acid to sodium hydroxide solutions. Curve 1, with 2 per cent gelatin; Curve 2, with 3 per cent gelatin.

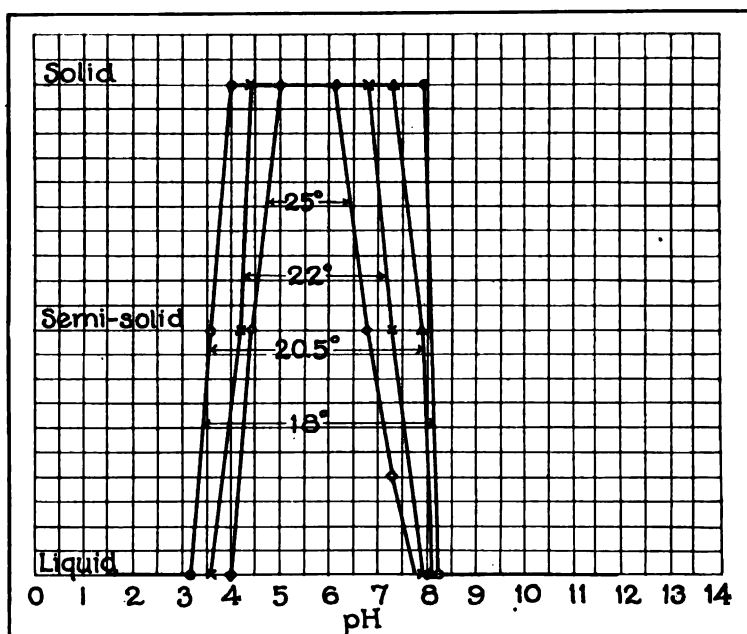


FIG. 7. Effect of temperature upon pH at which setting of gelatin takes place. Range 18–25°C. Medium, phosphoric acid 0.1 N through phosphates to sodium hydroxide (aqueous solution).

THE ZINC CONTENT OF SOME FOOD PRODUCTS.

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(From the Bureau of Chemistry, Department of Agriculture, Washington.)

(Received for publication, April 2, 1919.)

— 1 of the ash of man—

CORRECTION.

On page 100, Vol. XXXVIII, No. 1, May, 1919, line 19, for 20 cc. of creatinine solution, read 10 cc. of creatinine solution.

precipitations of the zinc sulfide were made from solutions of the chloride, instead of the sulfate, as usual, because, owing to the preponderance of calcium in the ash of milk, there seemed to be some danger of losing zinc through occlusion in the calcium sulfate from which the sulfate solution would have to be filtered off. The precipitation of zinc sulfide from a chloride solution, and its

¹ Lechartier, G., and Bellamy, F., *Compt. rend. Acad.*, 1877, lxxxiv, 687.

² Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313.

³ Javillier, M., Thesis, Univ. of Paris, 1908.

⁴ Weitzel, A., *Zentr. Physiol.*, 1914, xxviii, 766.

⁵ Breyer, F. G., in Scott, W. W., *Standard methods of chemical analysis*, New York, 2nd edition, 1917, 487.

filtration on the following day, were effected without difficulty in nearly all cases. All ashes were prepared in platinum, using an electric muffle. The details of the process are given below.

Preparation of Standard Zinc Solution.

1.2447 gm. of pure ignited zinc oxide (Kahlbaum) are dissolved in hydrochloric acid in a 1 liter volumetric flask. Ammonia is then added in such amount as to leave the solution clear and but faintly acid. The flask is now filled up to the mark with distilled water and shaken. For the turbidimetric estimation, a 10 cc. portion of this solution is diluted to 100 cc. in a volumetric flask. Each cc. of this solution contains 0.1 mg. of zinc.

Preparation of Ferrocyanide Solution.

34.8 gm. of pure potassium ferrocyanide (Kahlbaum) are dissolved in distilled water, and the solution is made up to 1 liter. The solution may have to be filtered, but need not be renewed for long periods, if kept in a dark place.

Precipitation of Zinc as Sulfide.

A given portion of the material in question is weighed into a platinum dish and carefully ashed at low heat, using an electric muffle. The ash is dissolved in hydrochloric acid and the solution transferred to a so called beaker flask of Kavalier Bohemian glass (the use of borosilicate glass of the Jena type should be avoided on account of the zinc content of these glasses). To the solution in the flask are added a few drops of methyl orange indicator, and then, in portions, dry sodium carbonate until the solution turns yellow. 50 per cent formic acid solution is now added drop by drop until the red color just reappears; then two or three drops are added to prevent precipitation of iron, the amount depending, of course, on the volume of the solution. The flask is now heated to near the boiling point and a lively stream of H_2S gas passed through the liquid until the latter has cooled completely. The flask is covered and allowed to stand over night.

The precipitate is collected on a small filter and washed with hydrogen sulfide water, slightly acidulated with formic acid. A clear filtrate should be obtained.⁶

Turbidimetric Estimation of Potassium Zinc Ferrocyanide.

The method employed differs in several respects from the one recently described by Meldrum⁷ for the estimation of zinc in water. The use of ordinary Nessler tubes was soon abandoned, since the estimation of slight differences in the turbidity, even when viewed against a black background, was found to give very uncertain results. By using the so called Nessler jar,⁸ a cylindrical vessel with an inside diameter of 32 mm., with lip and polished bottom, and graduated at 50 and 100 cc., the method can be put on an accurate basis. The determinations are made by placing the jar, which is filled up to the 50 cc. mark with the solution to be tested, on a clean sheet of print, preferably a table of figures, *e.g.* a table of logarithms. By looking down through the column of liquid, the print will appear more or less turbid depending on the amount of potassium zinc ferrocyanide held in suspension. If only jars with plane bottoms are used differences in turbidity may be readily seen which it would be quite impossible to distinguish by viewing the vessel from the side. By keeping in readiness a number of jars containing measured volumes of the standard zinc solution and using the same amount of acid and of ferrocyanide for each determination, the zinc content of a given solution can be quickly ascertained by comparison with these standards. In the analyses reported below, the amounts of acid and ferrocyanide contained in the final 50 cc. of solution were 3 cc. of concentrated hydrochloric acid and 5 cc. of the ferrocyanide solution in each instance. For accurate results the analyst will do best to concentrate his attention on one or two simultaneous determi-

⁶ In my own work, when investigating a new substance, I have always made a preliminary precipitation with hydrogen sulfide from an acid solution (1 part of concentrated HCl in 20 parts of solution) using a pressure bottle. But since there was nothing removed thereby in any case which would have interfered with the turbidimetric determination of zinc as described below, the first H₂S treatment is probably unnecessary.

⁷ Meldrum, R., *Chem. News*, 1917, cxvi, 295, 308.

⁸ See Eimer and Amend Catalogue C, 1913, No. 4152.

nations, especially since the whole procedure requires but a few minutes. The following are the details of the process.

The small filter holding the zinc sulfide is treated with 6 cc. of a hot solution of hydrochloric acid (1:1), the filtrate being poured back once to be finally collected in a Nessler jar. The filter is washed with distilled water until the liquid in the jar stands somewhat below the 50 cc. mark. Definite volumes of the standard zinc chloride solution are measured from a burette into three or four Nessler jars, into each of which water and 3 cc. of concentrated hydrochloric acid are also placed. The strength of the standard set of solutions is chosen so as to come near the amount of zinc expected in the sample. Thus, if 0.3 mg. is expected, the standard set may contain 2.5 cc., 3.0 cc., and 3.5 cc. of the standard zinc solution respectively in each jar. When all the jars are in readiness, 5 cc. of the potassium ferrocyanide solution are pipetted quickly into each, the liquid is brought to the 50 cc. mark with distilled water, shaken, and the jars are at once placed on the printed sheet for comparison.

Sources of Error, Sensitiveness, and Limitations of the Method.

Sources of error which should be guarded against are a loss of zinc during ignition and an incomplete precipitation of the zinc sulfide due to an excessive concentration of formic acid. Both these errors would cause low results. In general, provided no zinc is introduced with the reagents or from the glassware, there is little chance for obtaining high results with this method. With substances from which zinc is lost through reduction during the ignition, a strong acid digestion or a careful ashing with the addition of free sulfuric acid may be resorted to.

The turbidimetric method, as outlined, can be used directly only in cases where the amount of metallic zinc in the sample is below 0.5 mg. With larger amounts, the turbidity produced becomes so strong as to render invisible the printed characters on the sheet below the column of liquid. In such cases, provided the zinc content of the sample is still below 1 mg., it may be determined accurately by adding another 5 cc. of ferrocyanide solution, 3 cc. of hydrochloric acid, then diluting the contents of the jar to the 100 cc. mark with distilled water, mixing by pouring

into a second Nessler jar, and finally dividing the liquid equally between the two jars, with either one of which the reading may be obtained. If the zinc sulfide precipitate is heavy, it is necessary to collect the dissolved precipitate in a volumetric flask, and to use an aliquot portion for the determination.

The amount of zinc necessary to respond to the turbidimetric test is small, the complex potassium zinc ferrocyanide being highly insoluble. With solutions containing as little as 0.02 mg. of metallic zinc a perceptible turbidity develops within a few moments after adding the ferrocyanide solution.

The mixtures in the jars gradually turn more turbid on standing. For this reason the final mixture of the sample and the standard solution with which it is to be compared should be made up as nearly simultaneously as possible. With a solution of unknown zinc content, this may be done as follows: A series of Nessler jars, each holding 3 cc. of concentrated hydrochloric acid, a measured volume of the standard zinc solution, and water, are prepared. The first 5 cc. of ferrocyanide solution are now run into the solution to be tested. From the degree of turbidity which develops, the experienced analyst can judge at once the approximate zinc content of the solution. The second 5 cc. of the ferrocyanide solution are then quickly pipetted into a jar of the standard set containing somewhat less zinc than there appears to be in the sample. The final adjustment is made by comparing the two jars on a sheet of print and adding small amounts of standard zinc solution from the burette to the jar from the standard set until the turbidity of the latter equals that of the sample.

For the purpose of testing the accuracy of the method, some determinations were made on samples of skimmed milk to which measured amounts of standard zinc solution had been added. The results in Table I show that the method is sufficiently accurate for all practical purposes. If properly carried out, the experimental error should not be over ± 0.05 mg.

The determinations in Table II were made in order to ascertain what error in the results might be caused by the handling and washing of very small amounts of zinc sulfide precipitate in the average determination. Measured volumes of standard zinc solution were placed into flasks and diluted with water by an-

other chemist. The samples were then handed to me for analysis by precipitation as sulfide and subsequent turbidimetric determination. The results given in Table II show that no appreciable loss of zinc need occur during the filtration of the sulfide, the amount of zinc recovered being well within the ± 0.05 mg. limit which is to be allowed for the method in general.

TABLE I.
Analysis of Skimmed Milk Samples to Which Small Quantities of Zinc Chloride Solution Had Been Added.

Amount of sample.	Zinc content of sample.	Amount of zinc added.	Total zinc present.	Zinc found.
cc.	mg.	mg.	mg.	mg.
25	0.11	0.23	0.34	0.31
25	0.11	0.39	0.50	0.45
50	0.22	0.17	0.39	0.40
75	0.34	—	—	—

TABLE II.
Loss of Zinc Due to Handling and Washing of Zinc Sulfide Precipitate.

Sample No.	Zinc in sample.	Zinc found.
	mg.	mg.
1	0.15	0.13
2	0.05	0.04
3	0.11	0.09
4	0.15	0.18
5	0.20	0.18
6	0.30	0.26
7	0.20	0.17

Application of Method.

The method as outlined above was at first applied to samples of cow's milk; later a number of other food materials were included in the investigation. Zinc was found in nearly all of them, as shown by the results in Table III.

DISCUSSION.

The data contained in Table III are noteworthy in two respects. They illustrate not only the wide distribution of the element zinc in animal and vegetable products, but they also point

strongly to a certain nutritive function possessed by this element, the exact nature of which is not at present understood. Since the food as well as the bodies of men and animals normally contain small amounts of zinc, special feeding experiments with a zinc-free diet very likely would throw light on this question.

In the brief abstract of his work, Weitzel⁴ reports the constant presence of zinc in the urine and the feces of men. Working for over a month on three individuals, he found that from 2.7 to 12.8 mg. of zinc were eliminated daily with the feces. From the results of this author and those of Mendel and Bradley² it would seem that zinc is stored especially in the liver of animals, and the latter authors suggest that it might exert some influence on the respiratory functions of the blood. The animal used in their experiments was the mollusk *Sycotypus canaliculatus*, in the dry matter of which they found 1.7 per cent of zinc. They suggest, moreover, that all marine animals may contain this element in their tissues since sea water itself, according to Dieulafait,⁹ contains at least 2 mg. of zinc per cubic meter. From my own results in Table III it appears that oysters and agar-agar, both of which are products of the ocean, actually contain notable amounts of this metal.

Weitzel does not describe his method of investigation aside from saying that the zinc was weighed as oxide (presumably after ignition of the sulfide). As appears from his table, it was necessary to ash relatively large amounts of material, which constitutes a serious drawback to his method. His findings are nevertheless of great interest. He evidently was the first to make a quantitative determination of zinc in cow's milk and in eggs. My own findings give higher zinc values for these two products, but the differences between my results and those of Weitzel are not marked. For eggs, my own studies have revealed the fact that practically all the zinc in a hen's egg is contained in the yolk. This finding alone points strongly to the physiological importance of the element for the development of the young.

The zinc content of cow's milk was at first studied on samples of mixed market milk which is sold in bottles by several dealers in the District of Columbia. The zinc content of these samples, as

⁹ Dieulafait, L., *Compt. rend. Acad.*, 1880, xc, 1573.

TABLE III.
Zinc Content of Various Food Products.

	Amount of sample.	Amount of ash found.	Zinc (Zn.).		
			Found in sample.	In 1,000 gm. of fresh substance.	Calculated as per cent of ash.
	gm.	per cent	mg.	mg.	per cent
Tap water, Washington, D. C.....	500.00 cc.	0.0067	0.125	0.25	0.38
Yolk of egg (mixture of 3 yolks).....	16.6234	1.39	0.74	44.50	0.32
White of egg }.....	39.1229	—	Trace.	—	—
Egg shell }.....	6.4825	—	0.0	0.0	0.0
Yolk of egg }.....	17.6330	1.52	1.00	56.71	0.37
Whole egg (addition of above 3 parts).....	63.4430	—	1.00	15.76	—
Soy bean, dry.....	0.8421	3.62	0.10	0.12	0.33
Smooth pea, dry.....	4.9225	3.2 (sulfate ash.)	0.17	34.53	0.11
Carrot.....	26.1800	0.82	0.13	4.96	0.06
Bone ash.....	0.3598	—	0.14	—	0.01
Baker's yeast (8.07 per cent moisture).....	2.6515	7.65	1.10	414.86	0.54
Barley malt.....	1.8190	3.02	0.20	11.00	0.36
Barley, (Chevalier, Pacific Coast).....	2.6225	2.34	0.07	26.70	0.11
Corn (<i>Zea mays</i>), whole kernel.....	3.9620	1.09	0.10	25.24	0.23
“ “ kernels degerminated.....	5.0146	0.30	0.02	3.99	0.13
Wheat bran.....	2.2980	4.36	0.32	139.20	0.32
Wheat I, (Diets check, Va.).....	4.0088	1.78	0.34	84.80	0.48
“ II, (Fultz, Va.).....	4.0255	1.67	0.18	44.71	0.27
“ III, (Early Baart, Pacific Coast).....	4.6020	2.11	0.12	26.08	0.12
“ IV, (Marquis, S. Dakota).....	4.2201	2.22	0.20	47.40	0.21

Oat I, (Red oats, Cal.).....	4.0995	3.48	0.13	31.71	0.09
" II, (Abundance, S. Dakota).....	3.8610	2.79	0.125	32.38	0.12
" III, (Winter Turf, Va.).....	2.0254	3.13	0.10	49.37	0.16
" IV, (Canadian, Idaho).....	4.4073	3.66	0.17	38.57	0.10
Rye.....	5.2362	1.60	0.09	17.19	0.10
Rice, unhulled, Japanese.....	4.0956	5.93	0.06	14.65	0.03
Shred agar (Merck and Co.).....	5.8336	5.04	0.08	13.71	0.03
Gelatin (A. H. Thomas Co., No. 33).....	4.3799	2.13	0.12	27.40	0.13
Shucked oyster.....	14.6907	1.85	17.00	1,157.25	6.20
Skimmed milk.....	50.00 cc.	—	0.22	4.40	—
Mixed market milk:					
No. 1.....	150.00	—	0.60	4.00	—
" 2.....	100.00	—	0.43	4.30	—
" 3.....	100.00	—	0.56	5.60	—
" 4.....	83.00	—	0.40	5.00	—
" 5.....	83.00	—	0.35	4.20	—
" 6.....	83.00	—	0.32	3.90	—
" 7.....	83.00	—	0.31	3.80	—
" 8.....	83.00	—	0.31	3.80	—
" 9.....	83.00	—	0.35	4.20	—
" 10.....	83.00	—	0.33	4.00	—
" 11.....	83.00	—	0.32	3.90	—
" 12.....	83.00	—	0.30	3.60	—
Average of 12 samples.....		0.7 (estimated.)		4.20	0.06 (estimated.)

shown in Table III, ranged from 3.6 to 5.6 mg. per kilo, the average of twelve determinations being 4.2 mg. per kilo. The zinc content of the milk from individual cows was then determined. The samples were obtained from the St. Elizabeth Hospital Dairy in the District of Columbia. The cows were milked directly into Kavalier beaker flasks of zinc-free Bohemian glass; the analysis was begun upon reaching the laboratory. From the results obtained it appears that the zinc content of cow's milk varies somewhat in different animals and at different stages of the lactation period. It is evidently highest, about 5.5 mg. per liter, shortly after parturition, then for a long time remains constant at about 4.0 to 4.5 mg. per liter, to drop off markedly near the end of lactation. The first drop is illustrated by the results obtained with Cow 8 (see Table III). On the 7th day after parturition the milk of this cow contained 5.4 mg. of zinc per kilo, while 2 weeks later the value had fallen to 4.6 mg. per kilo.

Interesting results were obtained with samples of human milk, which were secured at the Freedmen's Hospital, Washington, D.C., from three mothers. A given volume of human milk evidently contains considerably more zinc than an equal volume of cow's milk; and it would appear that the zinc content of human colostrum is lower than that of later milk. These findings are of special interest in view of the fact that the total ash content of human milk is only about one-third as high as the ash content of cow's milk. From this and other evidence the conclusion may safely be drawn that zinc, like iron, is not present in milk as a simple inorganic salt, but that it enters into the composition of some organic milk constituent.

Finally I investigated the zinc content of the so called "neutralization precipitate" of cow's milk, which has been the subject of study by Osborne and Wakeman¹⁰ and by Palmer.¹¹ The neutralization precipitate was prepared in one instance from skimmed milk in the manner described by Palmer. The finely powdered substance when analyzed contained 7.62 per cent moisture and yielded 60.35 per cent ash. Its zinc content as shown in Table III is 674.31 mg. of zinc per kilo. With this preparation a confirmatory test was also made to be sure that the element in

¹⁰ Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1916-17, xxviii, 1.

¹¹ Palmer, L. S., *J. Assn. Offic. Agric. Chemists*, 1916, ii, 4.

question is actually zinc. A portion of the sulfide precipitate was carefully ignited. The resulting oxide was yellow while hot and white after cooling; this behavior is characteristic of zinc. In a second case, the neutralization precipitate was obtained by neutralizing the milk from one cow with ammonia, after removing the casein and albumin in the usual manner. 11.2345 kilos of whole milk yielded 65 gm. of neutralization precipitate (air-dry). The zinc determination on the latter gave 739.89 mg. of zinc per 1,000 gm. of substance, as shown in Table III. Hence the 65 gm., representing 11.2345 kilos of milk, contain 48.09 mg. of zinc, which is equivalent to 4.28 mg. of zinc per 1,000 gm., the normal value of milk. It is therefore evident that if the acidified serum of cow's milk is neutralized, practically all the zinc which the milk contains will come down with the "neutralization precipitate."

The normal occurrence of the element zinc in such vitally important animal products as milk and eggs, as well as in many other foods, deserves the close attention of the physiologist and the student of nutrition; especially since the quantities found are not at all negligible. The iron content of milk, which in spite of more than a century's research cannot be said to be definitely known, is probably not much, if at all in excess of the zinc content; and manganese has been shown by Nockmann¹² to be present in much smaller quantities. Moreover, it has been demonstrated that the amounts of these elements in milk are stationary, and that their quantity in the milk will not increase beyond a certain narrow limit upon increasing their proportion in the food of the animal. The same may be expected to hold for zinc in consequence of the well known selective function of the mammary gland. At any rate, it seems proper to consider the element zinc no longer as an unusual and unnecessary constituent of some few organisms, but as a regular and probably essential ingredient of living protoplasm.

Of the two cereals, wheat and oats, four different varieties were investigated of each. The zinc content of Wheat I is shown in Table III to be rather high. The low zinc content of the Washington tap water also deserves mention. In Weitzel's locality the zinc content of the water was more than nine times as high.

¹² Nockmann, E., *Molkereizeitung*, Hildesheim, 1914, xxviii, 419.

SUMMARY.

1. Zinc has been found and determined quantitatively in a number of food materials by using the turbidimetric ferrocyanide method.

2. In hen's eggs practically all the zinc is contained in the yolk, amounting to about 0.005 per cent of the latter. White of egg contains a mere trace of the metal at the most. The total amount present is about 1 mg. per egg.

3. The zinc content of ordinary market milk averages about 4.2 mg. of metallic zinc per kilo of milk.

4. The zinc content of the milk from individual cows varies somewhat in different animals. It is highest during the early part of the lactation period.

5. If the acidified serum of cow's milk is neutralized, practically all the zinc which the milk contains will be found in the "neutralization precipitate."

6. The zinc content of human milk is markedly higher than that of cow's milk in spite of the higher total ash content of the latter.

7. From its constant occurrence in the yolk of eggs as well as in cow's and human milk, it is inferred that the element zinc exerts an important nutritive function, the nature of which is not at present understood.

ZINC IN OYSTERS.

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United States Department of Agriculture.)

(Received for publication, March 28, 1919.)

In 1904 Bradley¹ reported the presence of zinc and also of copper in the liver of *Sycotypus canaliculatus*, a large carnivorous snail abundant through Long Island Sound, and in *Fulgur carica*, an allied mollusk. Several other forms of marine mollusks from the Sound, including the oyster, *Ostrea virginiana*, were examined with negative or doubtful results in all cases. Later Mendel and Bradley² found that in *Sycotypus* the blood and liver tissue alone contain zinc and copper. Mendel and Bradley² also observed that zinc was present in the common drill, *Urosalpinx cinerea*, but were unable to find appreciable amounts in the mussels, *Mytilus edulis* and *Modiola pheatula*, in "bloody clams," *Argina pexata*, in hermit crabs, *Eupagurus pollicaris*, in oysters, *Ostrea virginiana*, and in the common crab, *Cancer irroratus*. They clearly stated their belief, however, that other marine forms would probably be found to contain zinc, derived either directly from the sea water or, more probably, in accord with other familiar examples, picked out and retained by some simple organisms or plants, and thence reaching the carnivorous *Sycotypus* through a series of more complex and larger forms, such as the oyster, on which *Sycotypus* feeds. Moreover, ocean water is known to contain slight traces of the less common elements such as arsenic, copper, lead, and zinc. Dieulafait³ found that sea water contains about 2 mg. of zinc per cubic meter. Copper is also known to be present in sea water and in many marine animals. In 1908 Willard⁴ made a study of the copper content of oysters obtained from various districts on the Atlantic coast. He determined the copper by electrolytic deposition.

The results of an analytical search for metals in Tortugas marine organisms were reported by Phillips in 1917.⁵ Large amounts of copper were found in some of the specimens analyzed, and zinc in all except the

¹ Bradley, H. C., *Science*, 1904, xix, 196.

² Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313.

³ Dieulafait, L., *Compt. rend. Acad.*, 1880, xc, 1573.

⁴ Willard, J. T., *J. Am. Chem. Soc.*, 1908, xxx, 902.

⁵ Phillips, A. H., in *Papers from the Department of Marine Biology of the Carnegie Institution of Washington, Publication 251*, 1917, xi.

crayfish blood. Since the Tortugas Islands are far removed from any possible contamination of the sea water, zinc must be considered as a normal constituent of these forms. Manganese was present in all the specimens analyzed, but in varying amounts. The most remarkable occurrence, however, was that of lead in the liver of *Fasciolaria gigantea*, in quantity just sufficient to weigh, in a 20 gm. sample of dried liver, but quite enough to yield good qualitative tests.

In 1915 the presence of notable amounts of zinc in oysters was discovered almost simultaneously in two of the laboratories of the Bureau of Chemistry, U. S. Department of Agriculture. Each laboratory was working independently of the other and on quite different problems.

In the winter of 1915, Sale, in the Water Laboratory in Washington, discovered the presence and determined the quantity of zinc and manganese in samples of oysters from Chesapeake Bay. This work was done in connection with a study of the composition of the water of the Bay. At about the same time, but without knowledge of Sale's studies, the writers, working in the Denver Laboratory on the problem of the composition of oysters from polluted waters of the Atlantic coast, discovered zinc in astonishingly large amounts in each of the samples examined.

EXPERIMENTAL.

With the assistance of a number of the analysts of the Bureau of Chemistry laboratories in New York, Philadelphia, Boston, and Denver, the occurrence of zinc and also of copper in oysters from various localities on the Atlantic seaboard, the relation of zinc in oysters to the zinc content of the water in which they grew, and the ratio of zinc to copper in oysters were studied. The results of these studies are reported in the present paper. So far as is known, there have been published no other analyses of oysters or other shell fish that are used as food, with the exception of the Tortugas marine organisms already mentioned, showing the presence of zinc, arsenic, or other poisonous metals excepting copper. The analyses of Feldstein⁶ in the Denver laboratory, however, show that zinc is present in considerable amounts also in many

⁶ Unpublished investigations.

of the common edible marine animals such as clam, mussel, abalone, snail, shrimp, crab, and octopus.⁷

Identification of Zinc.—On account of the quantity of zinc reported by the analysts cited, it seemed important to establish the identity of the metal beyond question. To this end, an authentic sample of shucked oysters, on which a report had been made by Wichmann of 1,392 mg. per kilo, was subjected to a careful examination. Clearly positive reactions were obtained in every test. The solubility of the various salts, the color of the oxide, hot and cold, the color and solubility of the sulfide and of the ferrocyanide and phosphate were all strictly normal. Practically, the theoretical weight of anhydrous zinc sulfate was obtained from the oxide produced by the ignition of the sulfide precipitated from acetic acid solution. The same was true of the weight of pyrophosphate obtained by the conversion of the sulfate described above. The yield from 0.3792 gm. of oxide was 0.7509 gm. of anhydrous sulfate and 0.7083 gm. of pyrophosphate. The theoretical yield from zinc oxide would be 0.7521 gm. of sulfate and 0.7099 gm. of pyrophosphate.

Methods of Analysis.

No originality is claimed for the procedure followed in this study. Well known and reliable methods were adopted. The following details, developed to meet the requirements, were found to give dependable results.

1. *Preparation of Sample.*—At least a pint, representing the lot under examination, was thoroughly comminuted, using a form of grinder or chopper that did not cause a separation and loss of the fluid portion. Any liquid that separated was intimately mixed with the solid portion after grinding. Copper and zinc were determined in one weighed portion of the sample, using 100 gm. when practicable.

2. *Destruction of Organic Matter.*—This is a necessary preliminary operation for determining both zinc and copper. This was accomplished by the acid digestion process, using either a Kjel-

⁷ The octopus analyzed is the canned Japanese commercial article, species not determined.

dahl flask or a silica dish, preferably the latter. Results by both methods were found to agree.

100 gm. of the well mixed sample were weighed into a silica dish, 20 cc. of 25 per cent sulfuric acid added, and evaporated on the steam bath over night. The residue was then carefully heated and the excess of sulfuric acid slowly fumed off by placing the dish in front, but not inside of a hot muffle. The organic matter was thus readily oxidized without overheating. When the excess of sulfuric acid was removed the contents of the dish were moistened with concentrated nitric acid and gradually heated. Nitric acid was added in small quantities from time to time, followed by periods of heating inside the muffle at gradually increasing temperatures until the organic matter was destroyed. Care was exercised to control the temperature of the muffle below a dull red heat at first, and to add sufficient nitric acid to prevent flaming and possible loss of zinc. The nitric acid was added most conveniently drop by drop from a pipette directly to the hot material. This method is preferred for examining oysters and similar products because a large sample can be disintegrated and reduced to ash easily and quickly without the danger of losses by frothing or by breakage of apparatus. It is not feasible to destroy the organic matter directly by burning, owing to the danger of losing both copper and zinc by volatilization.

3. *Separation of Copper from Zinc.*—Copper was precipitated as sulfide from a slightly acid solution with hydrogen sulfide, filtered, and the filtrate reserved for the determination of zinc.

4. *Determination of Zinc.*—The acid filtrate contained zinc, iron, manganese, phosphates, etc. To determine zinc, the filtrate was treated with 1 cc. of nitric acid and evaporated to dryness and the residue dissolved in 50 cc. of a reagent of the following composition: ammonium chloride, 200 gm.; ammonia, 500 cc.; water, 750 cc. The solution was heated to boiling and treated with a few cc. of bromine water, then boiled 2 or 3 minutes, filtered, and the precipitate washed with a hot solution consisting of 100 gm. of ammonium chloride and 50 cc. of ammonia to the liter. The filtrate contained all the zinc originally present in the sample. The filtrate was neutralized with hydrochloric acid and an excess of 2 cc. of concentrated acid added. The solution was then diluted to about 200 cc. and titrated hot with

a standardized solution of potassium ferrocyanide equivalent to 2 to 3 mg. of zinc per cc., using a 5 per cent solution of uranium acetate on a spot plate as an "outside" indicator. Whenever it was necessary to make a series of zinc determinations the ferrocyanide solution was standardized using a pure zinc solution of known strength containing ammonium salts and hydrochloric acid in about the same proportion as in the sample under examination. This procedure is essentially the method described by Low.⁸ The details and precautions suggested in the text should be carefully observed. The results of analyses are stated in terms of mg. per kilo.

Copper may be determined in the sulfide precipitate described above by any approved method. Low's⁸ iodometric method gives reliable results and for several reasons is preferred.

Results of Analysis.

The results of the determinations of zinc in oysters by the method described are given in Tables II to VI, inclusive.

Table I contains the results obtained by Sale by a different method. The presence of zinc was not anticipated by him, so that the samples were reduced to ash as a rapid means of preparing them for analysis. A portion of the zinc may have been lost in the operation by reduction and volatilization. The zinc and manganese were precipitated together as sulfides and reprecipitated with sodium carbonate and weighed as oxides. The values for zinc were obtained by difference after subtracting the figure for manganese, which was determined colorimetrically.

In all the tables the results are given for each sample analyzed for the purpose of showing the individual variations. A statement of the averages would hardly suffice to show the true status of the matter.

From the data presented it is evident that zinc is present universally in oysters, or at any rate in those grown in the Atlantic waters along the coast of the United States.

The data tabulated also show that in oysters zinc is associated invariably with copper. No samples examined in the Bureau of

⁸ Low, A. H., Technical methods of ore analysis, New York, 5th edition, 1911.

TABLE I.
*Determination of Zinc in Shucked Oysters.**

Sample No.	Source.	Zinc in drained oyster flesh. mg. per kg.
	Shell stock from oyster house, said to be from	
8,905-H	Patuxent River, Md.....	111
8,906-H	Holland Point, Md.....	161
8,907-H	" " ".....	320
8,908-H	Rappahannock River, Va.....	102
8,909-H	" " ".....	82
8,910-H	Fishing Bay, Md.....	108
	Chester River:	
18,981-H	Bed 14, Queen Annes Co., Md.....	504
18,982-H	" 39, Kent Co., Del.....	247
18,984-H	" 43, " " ".....	394
18,985-H	" 46, " " ".....	459
18,989-H	" 4, Queen Annes Co., Md.....	274
18,991-H	" 64, Kent Co., Del.....	118
	Chesapeake Bay, lower end:	
20,520-H	Bed 15, Anne Arundel Co., Md.....	46
20,521-H	" 16, " " ".....	38
20,522-H	" 14, " " ".....	319
	Severn River:	
20,506-H	Bed 43, Anne Arundel Co., Md.....	58
20,507-H	" 23, " " ".....	124
20,509-H	" 27, " " ".....	620
20,515-H	" 23, " " ".....	217
20,517-H	" 45, " " ".....	143
20,519-H	" 45, " " ".....	26
18,945-H	Rhode River, Bed 72: Anne Arundel Co., Md.....	74
	West River:	
18,944-H	Bed 70, Anne Arundel Co., Md.....	186
18,947-H	" 81, " " ".....	281
	Patuxent River:	
18,908-H	Bed 3, St. Marys Co., Md.....	70
18,913-H	" 22, Calvert Co., ".....	186
18,994-H	" 22, St. Marys Co., ".....	171
18,995-H	" 22, Calvert Co., ".....	169
18,997-H	" 14, St. Marys Co., ".....	150
19,000-H	" 31, Calvert Co., ".....	61
—	Pink oysters (decomposed) shipped from South Norwalk, Conn.....	394
Maximum	620
Minimum	26

* Analyses made by J. W. Sale in the Water Laboratory, Bureau of Chemistry, 1914.

Chemistry have been found to be free from these metals. The amounts of metals present appear to have no direct relation to the body weight of the oyster nor to the quantity of ash-forming solids it contains. The presence of very large amounts of copper is usually revealed by a blue or bluish green coloration. Such oysters invariably contain large amounts of zinc, though not in uniform proportion to the copper. The blue coloration serves,

TABLE II.
*Determination of Arsenic, Copper, and Zinc in Oysters.**

Sample No.	Source.	Arsenic.	Copper.	Zinc.
		<i>mg. per kg.</i>	<i>mg. per kg.</i>	<i>mg. per kg.</i>
17,054-K	New York, N. Y.....	0.95	86.4	556.0
17,055-K	Crisfield, Md.....	None.	17.0	172.0
17,057-K	" ".....	0.77	17.8	133.7
17,058-K	" ".....	0.77	33.7	252.3
17,063-K	St. Michaels, Md.....	0.84	36.4	346.5
17,064-K	New Haven, Conn.....	1.24	72.9	1,115.0
17,065-K	New York, N. Y.....	1.16	75.1	541.0
17,066-K	Chincoteague, Va.....	1.47	11.9	171.0
17,069-K	New York, N. Y.....	0.98	98.5	692.3
17,069-K	(Drained meats).....	0.98	136.5	953.3
17,069-K	(Liquor).....	0.77	23.9	185.8
17,070-K	New York, N. Y.....	1.12	90.8	724.1
17,071-K	South Norwalk, Conn.....	1.12	158.8	1,168.5
17,072-K	" ".....	1.33	152.2	1,039.0
18,745-K	Boston, Mass.....	Not determined.	101.2	1,090.0
20,764-L	New York, N. Y.....	1.30	175.3	1,302.5

* Analyses made by H. J. Wichmann, in the Denver laboratory, January, 1915.

therefore, as a reliable indicator of the presence of large amounts of these metals. Indeed, the oysters from certain localities, such as Perth Amboy, New Jersey, and New York Harbor, have gained unfavorable notoriety because of the amounts of copper they contain, frequently excessive enough to impart to them a strong turquoise blue color and a metallic flavor, and to yield a decided copper coating to bright iron when boiled with it in dilute acid. Since the amounts of copper and zinc found in apparently nor-

TABLE III.
Determination of Amounts of Copper and Zinc in Shucked Oysters.*

Sample No.	Source.	Copper.		Zinc.	
		Drained oysters.	Liquor.	Drained oysters.	Liquor.
		mg. per kg.	mg. per kg.	mg. per kg.	mg. per kg.
B-812	Jamaica Bay, N. Y.	48.8	12.8	876	60.0
B-814	" "	92.8	11.2	540	52.0
B-816	" "	57.6	8.0	848	16.0
B-818	" "	80.0	11.2	616	8.0
B-820	" "	83.2	14.4	280	Trace.
B-822	" "	64.0	11.2	648	76.0
Maximum		92.8		876	
Minimum		48.8		280	
B-840	Great South Bay, off West Sayville, N. Y.	67.0	3.0	646	Trace.
B-842	" "	51.0	1.0	598	4.0
B-844	" "	161.0	4.0	1,102	11.0
B-846	" "	87.0	5.0	742	Trace.
B-848	" "	41.0	Lost.	534	Lost.
B-849	" "	94.0	5.0	701	10.0
B-850	" "	29.0	4.0	429	5.0
Maximum		161.0		1,102	
Minimum		29.0		429	

* Analyses made by Louis D. Elliott, in the New York laboratory, 1915.

B-851	Raritan Bay.	154.0	9.0	912	85.0
B-853	"	128.0	4.0	768	71.0
B-852	Sandy Hook.	167.0	7.0	1,082	57.0
B-854	"	141.0	5.0	912	69.0
A	Bridgeport, Conn., Bed 804.	444.2	21.8	1,088	70.4
B	" " 831.	283.5	16.6	828	67.2
C	" " 718.	285.2	11.5	280	33.6
D	" " 710.	370.1	16.6	880	64.0
E	" " 749.	255.2	17.9	960	99.2
A	Milford, " 674.	283.5	19.2	920	81.6
B	" " 657.	261.5	11.5	1,184	67.2
C	" " 607.	371.5	15.4	1,176	81.6
Maximum	444.2		1,184	
Minimum	255.2		280	
A	New Haven, Conn.	539.0	25.0	1,363	56.0
B	" "	257.0	8.0	1,760	37.0
C	" "	244.0	13.0	1,357	69.0
D	" "	270.0	16.0	1,475	84.0
E	" "	308.0	19.0	1,411	83.0
F	" "	180.0	13.0	870	94.0
Maximum	539.0		1,760	
Minimum	180.0		870	

TABLE III—*Continued*

Sample No.	Source.	Copper.		Zinc.	
		Drained oysters.	Liquor.	Drained oysters.	Liquor.
		mg. per kg.	mg. per kg.	mg. per kg.	mg. per kg.
A	Vicinity Stony Creek, Conn., Flying Point.....	124.0	6.0	870	39.0
B	" " " Grass Rock.....	131.0	4.0	714	24.0
C	" " " near Cut-in-Two Island.....	120.0	5.0	749	52.0
D	" " " between Waylands and Davis Island.....	124.0	6.0	982	79.0
E	" " " between Pot and Money Islands.....	146.0	7.0	1,014	89.0
F	" " " between High and Crib Islands.....	101.0	6.0	755	40.0
G	" " " northeast of Governor's Island.....	86.0	6.0	717	74.0
H	" " " northeast of Roger's Island.....	94.0	5.0	595	67.0
Maximum		146.0		1,014	
Minimum		86.0		595	
A	South Norwalk, Conn.....	172.0	20.0	1,315	108.0
B	" " " Town Bed.....	262.0	10.0	920	94.0
C	" " " Bed 1,078.....	290.0	10.0	1,792	80.0
D	" " " " 1,025.....	371.0	13.0	2,298	69.0
E	" " " " 1,118.....	318.0	13.0	1,789	102.0
F	" " " " 1,152.....	224.0	10.0	1,488	38.0
G	" " " Town Bed.....	179.0	12.0	1,798	107.0
I	" " " " 42.....	64.0	3.0	778	62.0
J	" " " Bed 1,048.....	203.0	24.0	1,894	83.0
K	" " " Tallmudges Bed.....	173.0	5.0	1,667	82.0
L	" " " mouth of Saugatuck River.....	132.0	6.0	1,331	32.0
Maximum		371.0		2,298	
Minimum		64.0		778	

3,032-K	Chesapeake Bay, Governor's Run.....	30.0	Not determined.	714	Not determined.
3,033-K	" " Lumps.....	181.0	"	1,162	"
3,034-K	" " Bodkin Point, Md.....	214.0	"	1,309	"
3,035-K	" " Swan Point, Md.....	179.0	"	1,197	"
3,036-K	" " Crab Alley Creek.....	59.0	"	461	"
3,037-K	" " Hoy Bay.....	101.0	"	512	"
3,038-K	" " Holland Point, Md.....	76.0	"	755	"
3,039-K	" " Chester River, Md.....	111.0	"	829	"
3,040-K	" " West River, Swan Point, Md.....	111.0	"	778	"
3,041-K	" " Port Tobacco, Md.....	101.0	"	624	"
3,042-K	" " Fishing Bay, Md.....	28.0	"	170	"
3,043-K	" " Herring Bay, Md.....	68.0	"	810	47.0
3,044-K	" " mouth of Patuxent, Md.....	38.0	"	381	Not determined.
3,045-K	" " Chester River, The Cliffs.....	88.0	"	522	"
	Maximum.....	214.0		1,309	
	Minimum.....	28.0		170	
346-K	Potomac River, near Coles Point, Va.....	38.0	Not determined.	355	Not determined.
347-K	Pocomoke Sound.....	52.0	"	256	"
348-K	Potomac River, off Point Lookout, Md.....	48.0	Trace.	182	"
349-K	" " near Blackstone Island, Md.....	54.0	"	237	40.0
351-K	Blackstone Island, Md.....	72.0	2.5	250	11.0
352-K	Jackson's Creek, Westmoreland Co., Va.....	46.0	Not determined.	307	Not determined.
3,121-K	Chesapeake Bay.....	20.0	"	189	"
3,122-K	South of Blackstone Island, Md.....	52.0	1.6	186	11.0
	Maximum.....	72.0		355	
	Minimum.....	20.0		182	

mal oysters from different localities vary so widely, and since there seems to be no uniformity in the ratio between their copper and their zinc content, it would seem that oysters are capable of taking up amounts of these metals beyond their immediate physiological needs.

There appears, moreover, to be no direct, uniform ratio between the quantity of copper and zinc in the oysters and the amount in the sea water in which they are found, although it is true, in general, that oysters contain larger proportions of the

TABLE IV.
*Determination of Copper and Zinc in Shucked Oysters.**

Sample No.	Source.	Copper in drained meats.	Zinc in drained meats.
		mg. per kg.	mg. per kg.
498-K	Bivalve, N. J.....	30.9	779
499-K	Maurice River, N. J.....	17.0	472
500-K	Parker's Bay, Stockton, Md.....	8.8	146
2,802-K	Johnson's Bay, Md.....	7.6	175
2,803-K	Franklin City, Va.....	8.8	153
2,804-K	West Sayville, L. I., N. Y.....	30.2	507
2,805-K	Maurice River, N. J.....	15.1	650
2,806-K	Baldwin, L. I., N. Y.....	6.3	562
2,807-K	Chincoteague, Va.....	18.8	201
2,808-K	Girdle Tree, Md.....	19.1	168
Maximum		30.9	779
Minimum		6.3	146

* Analyses made by Clarence L. Black, in the Philadelphia laboratory, March, 1915.

metals when grown in sea water highly contaminated with metallic wastes from smelters and other factories. The data presented in the tables show this clearly; for example, oysters dredged along the New Jersey coast near Perth Amboy and from waters farther north in New York Harbor. A notable exception to this general rule is seen in the samples collected from the vicinity of Savannah, Georgia, and analyzed in the New York laboratory (Table III, Samples 3,307-12-K). Sample 3,307-K, taken from the ocean about 80 miles south of Savannah, contained approximately twice as much copper and zinc as two other

samples (Nos. 3,308-K and 3,310-K) from beds in Savannah Harbor.

In order to secure data on the source of the zinc, the Bureau of Chemistry laboratory at New York analyzed two samples of sea water, and Feldstein, of the Bureau of Chemistry laboratory at Denver, analyzed eight samples of vegetation and organic matter that came up in the dredges with the oysters from beds

TABLE V.
*Determination of Copper and Zinc in Shucked Oysters.**

Sample No.	Source.	Copper in drained meats.†	Zinc in drained meats.‡
		mg. per kg.	mg. per kg.
2,741-K	Opposite Sayville, L. I., N. Y.	105	963
2,742-K	Bridgeport Gut, Conn.	362	1,050
2,745-K	Seaconet, Mass.	82	1,206
2,746-K	Wickford, R. I.	68	701
2,747-K	Waquoit, Mass.	34	872
2,748-K	Wellfleet, "	82	1,220
2,749-K	Cotuit, "	28	762
2,750-K	Bluepoint, L. I., N. Y.	26	658
2,756-K	Eastham Beds, Wellfleet, Mass.	16	964
2,757-K	India Neck, Mass.	18	984
2,761-K	Warwick Bed, Narragansett Bay, R. I.	62	400
2,762-K	Hyde Hole, " " "	94	392
2,763-K	Rumstick Bed, " " "	54	608
Maximum		362	1,220
Minimum		16	398

* Analyses made by C. W. Harrison, in the Boston laboratory, 1915.

† Copper determined electrolytically.

‡ Zinc weighed as oxide.

along the coast of Cape Cod (Table IV). In the sample of water taken from Great South Bay, 1 mile off-shore from West Sayville, New York, no copper or zinc in 3,500 cc. was found, though both metals were found in oysters from that region (Table III). In water from Lupatcong Creek, a tributary of Raritan Bay, Keyport, New Jersey, a trace of copper but no zinc was found in 4,000 cc. In two samples of oysters from Raritan Bay, the New York laboratory found respectively 154 and 128 mg. of copper per kilo of the drained oyster meat, and

912 and 768 mg. of zinc. The results of the analyses of the material brought up by the oyster dredges are given in Table VI. The samples were obtained personally by one of the inspectors connected with the Boston laboratory, so that their authenticity is

TABLE VI.

*Determination of Amounts of Copper and Zinc in Shucked Oysters and Marine Debris.**

Sample No.	Source.	Oysters. Meats and liquor combined.		Vegetation and debris accompanying the oysters in the dredge.†	
		Copper.	Zinc.	Copper.	Zinc.
		mg. per kg.	mg. per kg.	mg. per kg.	mg. per kg.
781	Wellfleet, Mass.....	14	705	Slight trace.	89
782	" "	10	772	" "	84
783	" "	16	224	No sample.	No sample.
784	" "	131	1,026	" "	" "
785	" "	26	946	Slight trace.	50
786	Cotuit, Mass.	34	877	" "	108
787	" "	8	477	None.	47
788	" "	Not ana- lyzed.		Slight trace.	54
789	Osterville, Mass.....	18	529	Trace.	56
790	" "	24	614	"	106
Maximum		131	1,026		108
Minimum		8	224		47

* Analyses made by L. Feldstein, in the Denver laboratory, June, 1917.

† The water from which these oysters were dredged is probably as free from pollution as any where oysters are produced commercially. Wellfleet is on the west coast of Cape Cod, and Cotuit and Osterville are on the south coast of Barnstable County, all remote from any large city or metallurgical works.

definitely established. The amount of metal in the organic matter itself is doubtless considerably larger than that indicated by the figures recorded because a quantity of sand, bits of shell, and other debris with which it was associated were weighed in with each sample analyzed.

Biological Significance of Zinc in Oysters.

In the course of this study, the writers have made no attempt to determine the physiological or biological significance of the zinc found in oysters nor to correlate the chemical data with the physiology of the mollusks. However, the finding by Feldstein of zinc in the organic matter which was dredged up with oysters may be regarded as supporting the suggestion made by Mendel and Bradley that this metal reaches the mollusk as a constituent of some one or other of the organisms upon which the mollusks feed. If this hypothesis can be substantiated further, it may furnish the key to the explanation of the lack of correlation observed in this investigation between the zinc and copper content of the oysters, and the zinc and copper content of the sea water in which the oysters grew. It may be that the oysters are rich in zinc when their food contains a considerable number of organisms, whatever they may be, that are rich in zinc, irrespective of the zinc content of the sea water, and one would then expect that the zinc content of the oyster is an indication of the character of its food rather than of the character of the sea water in which it grows. Mendel and Bradley⁹ showed that the zinc present in the blood and liver of *Sycotypus* is undoubtedly combined with protein much as copper is combined in hemocyanin; and they have suggested the name "hemosycotypin" for the compound. In oysters it is probable that the zinc also occurs in the blood as well as in the body substance since the New York and Denver laboratories invariably found zinc, as well as copper, in the liquor drained from the oysters examined. The quantity of blood of the animal comprised in the liquor is variable and not readily determined, but it is probably always present to some extent in the drained liquid as a result of injuries to the oysters when shucked. Whether or not the zinc in oysters is combined with protein as in "hemosycotypin" was not investigated in the present study. Since, however, it has been shown that zinc is found in marine animals more commonly than supposed by Mendel and Bradley, it might be well to substitute some more general term, such as hemocuprazin or hemozincin, for the term hemosycotypin suggested by them.

⁹ Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1906-07, xvii, 167.

SUMMARY.

Zinc is present universally in oysters, at least in those grown in Atlantic waters.

There is no direct relation between the zinc content and the body weight of the oysters, nor uniformity of ratio of zinc to copper, nor correlation between the zinc content of the oysters and the water in which they grew.

The vegetation and organic matter dredged up with oysters in the single locality studied contained notable quantities of zinc and in some instances traces of copper.

As in *Sycotypus*, zinc is probably always associated with copper.

It seems probable that zinc, as well as copper, can be absorbed and retained in the tissues of the oysters in quantities far in excess of functional requirements, especially in oysters grown in waters badly polluted with metallurgical and factory wastes.

The writers gratefully acknowledge the assistance rendered by Carl L. Alsberg, Chief of the Bureau of Chemistry, and by Clarence L. Black, Leonard Feldstein, Louis D. Elliott, and C. W. Harrison, analysts, respectively, in the Philadelphia, Denver, New York, and Boston laboratories of the Bureau of Chemistry, also by the Chemist in Charge and the Inspector of the Boston laboratory in securing authentic samples.

THE NUTRITIVE VALUE OF YEAST PROTEIN.*

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The idea of the use of yeast as a source of food protein for man and the higher animals is not a new one; it has, however, been given renewed emphasis by the exigencies of the food situation during the war. Yeast is a highly nitrogenous by-product of the fermentation industries which heretofore has been largely wasted. It also presents the possibility of a synthetic production of protein from exceptionally simple compounds independent of seasons and crops.

More recently the remarkable efficacy of the ordinary yeasts used in the manufacture of alcoholic beverages and also in bakeries in supplying water-soluble vitamins has been demonstrated.¹ This property must be regarded as something apart from the nutrient value of the yeast protein as such.

In the case of both adult men and animals the utilization of the nitrogenous components of yeast as judged by the "coefficient of digestibility" and the nitrogen balance has repeatedly been reported as good, although the statements on this point are not

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ The earlier literature of the subject is reviewed in our paper on The Role of Vitamins in the Diet, *J. Biol. Chem.*, 1917, xxxi, 149. See also Hawk, P. B., Fishback, H. R., and Bergeim, O., *Am. J. Physiol.*, 1919, xlviii, 211. For the use of yeast in the diet of growing mice see Thompson, H. B., and Mendel, L. B., *ibid.*, 1918, xlv, 431.

uniformly unanimous.² For example, Funk, Lyle, and McCaskey³ assert: "A large part of the yeast nitrogen apparently has no food value. It is badly assimilated."

The nutritive needs of a growing animal make the most exacting demands for physiologically adequate protein; hence feeding experiments during growth are particularly advantageous for determining the dietary usefulness of nitrogenous nutrients. We are not aware of any records of prolonged growth when yeast was used as the sole source of protein. Funk, Lyle, and McCaskey³ state that "young rats can live on yeast as the sole nitrogen source for quite a long while, although it has not yet been proven whether they can subsist on it indefinitely."

The actual protocols recorded by Funk⁴ are neither convincing nor conclusive. One of the two experimental rats receiving its nitrogen in the form of yeast died with edema after 44 days. The other appears to have grown normally for 52 days. Funk states (p. 9) without submitting further evidence: "Yeast cannot be regarded as a good substitute for protein. There is even a slight indication of a toxic action."

We have kept rats successfully for more than a year covering the period of growth, upon a diet in which yeast furnished the sole source of nitrogen as well as water-soluble vitamine. The food mixtures consisted of:

	Per cent	Per cent
Dried yeast.....	30*	40†
Salt mixture‡.....	4	4
Starch.....	43	33
Butter fat.....	9	9
Lard.....	14	14

* Yeast obtained from the Schlitz Brewing Co., Milwaukee, Wis.

† Yeast obtained from the Hinckel Brewery Co., Albany, N. Y.

‡ For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

The growth curves are shown in the chart.

² For a bibliography of the subject see Hawk, P. B., Smith, C. A., and Holder, R. C., *Am. J. Physiol.*, 1919, xlviii, 199. Funk, C., Lyle, W. G., and McCaskey, D., *J. Biol. Chem.*, 1916, xxvii, 173. Schill, E., *Biochem. Z.*, 1918, lxxxvii, 163.

³ Funk, C., Lyle, W. G., and McCaskey, D., *J. Biol. Chem.*, 1916, xxvii, 173

⁴ Funk, C., *J. Biol. Chem.*, 1916, xxvii, 1 (see p. 9, Experiment 6).

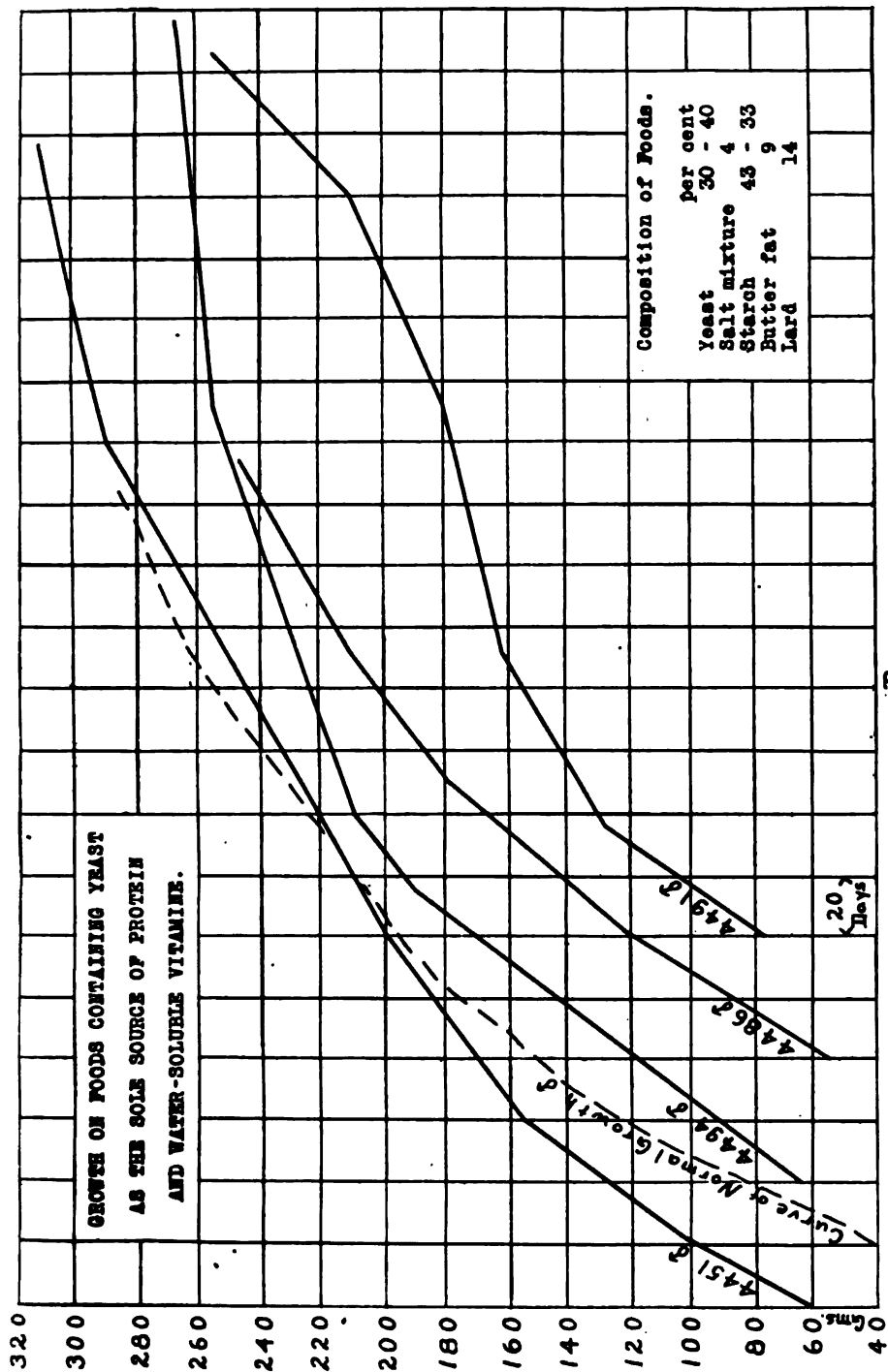


CHART I.

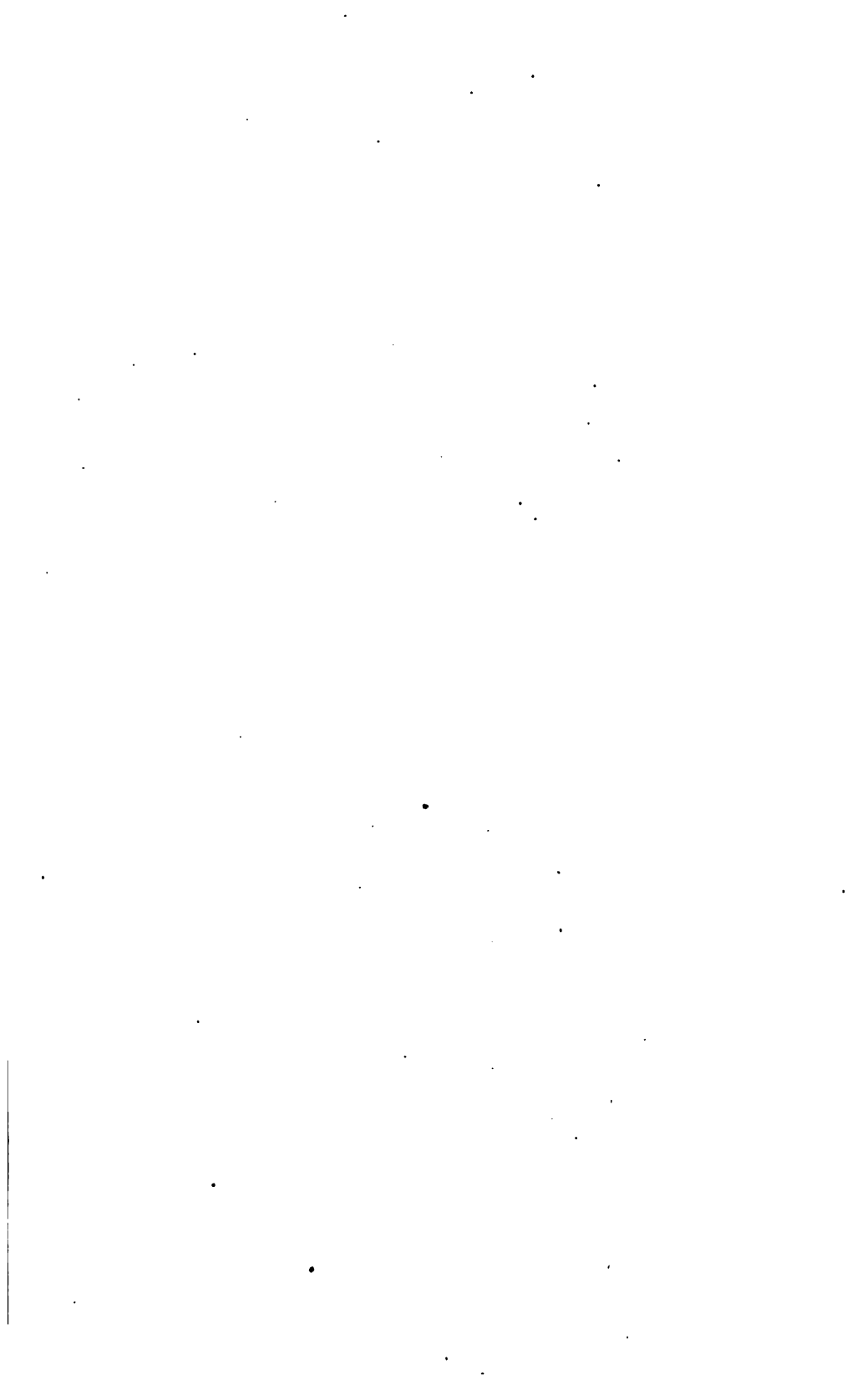
Inasmuch as a not inconsiderable part of the nitrogen of the yeast is non-protein nitrogen, represented by such compounds as nucleic acid and its derivatives, the actual protein concentration of the successful diet must have been even less than 18 per cent. An estimation of the digestibility of the food, tested by comparison of the feces with the intake showed a nitrogen "utilization" in several cases of 74 to 83 per cent.

In other experiments in which yeast was used solely as a source of water-soluble vitamine, it had been noticed that although the animals thus fed had grown vigorously to adult size they were, with very few exceptions, found to be sterile. Some of these rats were examined for us by Dr. Donaldson of the Wistar Institute, who reported that the testes showed an absence of germ cells. Other investigators have described similar conditions among animals fed on diets deficient in water-soluble vitamine.⁵

To determine whether this sterility was due to a deficiency in vitamine, or to some other property of the yeast, the rats which had grown to full adult size on a diet in which yeast composed 30 to 40 per cent of the ration, and which therefore contained an abundance of water-soluble vitamine, were mated with vigorous females which had grown up on a normal mixed diet. Two of the animals proved to be fertile but produced inferior young; while two of the other yeast-fed rats failed to breed. The latter were sent to Dr. Donaldson who kindly had them examined for us. He reported that they were somewhat heavy for their body length, the refractive index of the serum was below normal, and the organs were about normal, except the testes which were only one-quarter to one-third the normal size. These were not examined histologically, but a gross inspection indicated that the germ cells had been destroyed. Although the number of observations is far too small to justify final conclusions, the fact that two out of four of the rats which were long fed on very large quantities of yeast bred, indicates that infertility on diets in which only a small quantity of yeast supplied the vitamine was not caused by the yeast *per se*.

⁵ Drummond, J. C., *Biochem. J.*, 1918, xii, 25. Funk, C., and Douglas, M., *J. Physiol.*, 1913-14, xlvii, 475.

The fact that animals tolerate such large quantities of a food product so unique as is yeast in its chemical make-up seems to exclude the presence of a toxic factor. Consequently there is little occasion to fear any detriment from such doses of yeast as are employed for therapeutic purposes.



STUDIES ON ENZYME ACTION.

XVII. THE OXIDASE, PEROXIDASE, CATALASE, AND AMYLASE OF FRESH AND DEHYDRATED VEGETABLES.

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(Received for publication, April 16, 1919.)

Outline of Investigation.

The question of food hormones has become prominent in recent years in connection with the development of certain pathological conditions due to the lack of the chemically unknown constituents in diets apparently adequate in protein, fat, carbohydrate, mineral components, and calories. Under the term food hormones may be included vitamins, antiscorbutic property, growth-producing property, etc. Considerable evidence is being accumulated in regard to the treatment of foodstuffs which results in the loss of one or more of these food hormones. Recently published work on the loss of the antiscorbutic property for guinea pigs of certain vegetables on dehydration indicates that simple chemical or physical treatment may cause the destruction of these vitally important factors.¹ Until positive evidence to the contrary is forthcoming, the changes in the food hormones which take place on their "inactivation" may be considered to be chemical in character. The same standpoint will be taken with regard to their chemical nature as

¹ Holst, A., and Frölich, T., *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1; 1913, lxxv, 334. Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141. Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 487. Givens, M. H., and Cohn, B., *ibid.*, 1918, xxxvi, 127. Delf, E. M., and Skelton, R. F., *Biochem. J.*; 1918, xii, 448. Givens, M. H., and McClugage, H. B., *J. Biol. Chem.*, 1919, xxxvii, 253.

was taken in the chemical study of enzymes.² Food hormones are not considered necessarily to be chemical individuals. It is sufficient to ascribe more or less definite food hormone properties to certain groupings in the molecules, possibly protein, and the loss of such properties to a change (possibly tautomerism or rearrangement) in such groupings.

The changes in foodstuffs which result in different nutritive values do not appear in the ordinary chemical analyses. In order to obtain if possible some evidence of such changes, and also to follow any chemical changes taking place in such simple treatments as dehydration, this series of studies was undertaken. It includes a general enzyme study, a study of certain vegetable proteins, such as the determination of their isoelectric points, etc. (Cohn and coworkers), and an attempt to follow carbohydrate changes on dehydration. This paper will take up the results of the enzyme studies.

Changes in enzyme action (activation as well as inactivation) may be brought about by comparatively simple treatments. Changes in nutritive values of foodstuffs may also result from simple treatments. It is not assumed that the two sets of phenomena due to enzymes and to food hormones are due to the same chemical grouping or even molecule. It was considered that a general study of the enzymes of a number of vegetables would give results which, when compared with the physiological actions of the same vegetables would show similarities and differences which might aid in throwing light on the chemical nature of the substances involved in the various actions.

The enzymes oxidase, peroxidase, catalase, and amylase were studied in potatoes, tomatoes, carrots, white and yellow turnips, and cabbage. Fresh, air-dehydrated, and vacuum-dehydrated vegetables were used.

HISTORICAL.

Oxidases have been found to occur in many vegetables. There is no one method or reagent for testing for their presence. A number of different reagents have been used, such as various phenols, amines, etc. Peroxidases are almost universally dis-

² Falk, K. G., *Proc. Nat. Acad. Sc.*, 1916, ii, 557; *J. Biol. Chem.*, 1917, xxxi, 97; *Science*, 1918, xlvii, 423.

tributed in plants. Just as with oxidases, there is no general test for them. Catalase also is widely distributed in a number of different plants.

Quantitative measurements of the amounts of these enzymes, especially catalase, have been made at different times. The effect of the hydrogen ion concentration on their actions does not seem, however, to have been studied to any extent. Attempts at purifying the enzymes have also been described.

Although amylase has not been studied in many vegetables or plants, the work of Sherman and his associates³ on malt, pancreatic, and *Aspergillus oryzae* amylases has thrown much light on the conditions for their actions and the properties of highly purified materials. A careful study on potato amylase was published recently by Doby.⁴

Preparation of Materials.

The fresh vegetables were scraped (carrots and turnips), peeled (potatoes), or in the case of cabbage the outer leaves removed. They were then weighed, ground finely in a meat chopper, and squeezed through muslin by hand, until as much extract as possible was obtained. The tomatoes were cut and then squeezed similarly. The extract was strained through fresh muslin a second time to remove the coarser particles which had been forced through the first muslin. The residue was weighed and its moisture content determined by drying to constant weight at 100°. The volume of the extract was measured and its hydrogen ion concentration determined.

The air-dehydrated vegetables were prepared by blowing a current of heated air (40–65°) over the vegetable cut in thin slices for 18 to 60 hours until the substances were sufficiently dry. Some preparations (California) were bought on the open market. The vacuum-dehydrated products were prepared by heating the sliced vegetables at 55–65° at 40 to 50 mm. pressure for 8 to 12 hours. Moisture was determined in these products by drying to constant weight at 100°, nitrogen by the Kjeldahl method, and ash by ignition to constant weight.

³ Sherman, H. C., and associates; numerous papers in *J. Am. Chem. Soc.*, 1910–1919.

⁴ Doby, G., *Biochem. Z.*, 1914, lxxvii, 166.

After considerable experimentation, the following method of testing the dehydrated vegetables was adopted. To 14 gm. of dehydrated vegetable were added 180 cc. of water (the average amount lost on dehydration) at 20-25°. The mixture was allowed to stand for 20 minutes and then passed through the food chopper. The chopped vegetable was then returned to the water in which it had been soaked, ground with it in a glass mortar and allowed to extract for 30 minutes, squeezed through muslin, and treated as with fresh vegetables. This part of the investigation dealt mainly with enzymes. In attempting to prepare a juice from the dehydrated vegetables comparable to fresh juice, it was found that no more enzyme was extracted by treatment in the manner described for 2 hours or even longer than after 30 minutes treatment. This juice after longer extraction when tested with the solid residue re-added did not give as strong enzyme tests as the dehydrated vegetable ground in a mortar for a shorter period of time with water and tested immediately. Apparently the enzymes were not extracted as readily from the dehydrated products as from the fresh, and if left in contact with the water for longer periods of time deteriorated or lost their activity to some extent. Differences were found as well in the enzyme properties of products dehydrated by different processes, as will be pointed out later.

The properties of the dehydrated vegetables and of the juices prepared from fresh and dehydrated products may be summarized briefly. The appearance of the vacuum-dehydrated product was in every case superior to that of the air-dehydrated; better color, texture, etc. The chemical analyses did not show any definite differences. The moisture contents varied between 7 per cent and 20 per cent (highest with tomato preparations) for the various substances. No regularity was apparent except that longer dehydration yielded more anhydrous products. The ash percentages varied similarly in a more or less irregular manner. As these variations were apparently accidental, the results will not be given in detail.

The hydrogen ion concentrations of the juices were determined colorimetrically, using the Clark and Lubs indicators⁵ chiefly and also their solutions as standards. The indicators used included dibromocresolsulfonephthalein, dibromothymolsulfonephthalein, phenolsulfonephthalein, o-cresolsulfonephthalein, phenolphthalein, thymolsulfonephthalein, thymolphthalein, methyl orange, cochineal, methyl red, and rosolic acid.

⁵ Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.

Methods of Testing for Enzymes.

The following reagents were used.

For oxidase.—Röhmman-Spitzer reagent.⁶ 1 per cent aqueous p-phenylenediamine hydrochloride and 1 per cent α -naphthol in 50 per cent ethyl alcohol, kept separately and equal volumes mixed just before using. 5 drops of reagent were added to 2 cc. of juice or mixture and the color developed in $\frac{1}{2}$ minute and in 15 minutes was noted. A very faint pink color was marked 1 (+ or -) in the tables, a deeper shade 2, and a deep red-purple 3. This reagent is more delicate than guaiaconic acid or α -naphthol and was therefore adopted for oxidase. Its color could also be more readily detected through the natural color of the juice.

For peroxidase.—1 per cent α -naphthol in 50 per cent ethyl alcohol. 5 drops of this solution and 2 cc. of 3 per cent hydrogen peroxide solution at the same hydrogen ion concentration as the mixture tested were added to 2 cc. of the juice or mixture, and the color developed in $\frac{1}{2}$ minute and in 30 minutes was noted. The first pink or lavender was 1 (+ or -), a deeper lavender 2, a deep blue-purple 3. Blank solutions gave no test with the reagent in 1 hour at pH 2 to 11. The Röhmman-Spitzer reagent was found to be too delicate for peroxidase, differences at different hydrogen ion concentrations and with fresh and dehydrated products not being readily distinguishable.

For catalase.—Evolution of gas and production of foam on addition of hydrogen peroxide in peroxidase test were noted and indicated as before.

It was found that with dehydrated vegetables, 0.5 gm. ground in a mortar with 7 cc. of distilled water, transferred to a test-tube and tested for these enzymes, frequently showed greater actions than a mixture of the juice prepared as described from dehydrated vegetables plus the solid residue from the juice.

For amylase.—The Lintner method,⁷ modified slightly for the purposes in view, was used. An experiment consisted of a series of test-tubes each containing 0.5 cc. of the vegetable juice or mixture and 2 cc. of freshly prepared (Lintner) starch solution.

⁶ Röhmman, F., and Spitzer, W., *Ber. chem. Ges.*, 1895, xxviii, pt. 1, 567.

⁷ Lintner, C. J., *J. prakt. Chem.*, 1886, xxxiv, 378.

Immediately after making up the mixtures (for the blanks) or after 2 hours incubation at 37° (or other time interval) varying amounts of Fehling solution differing by 0.5 cc. were added to the test-tubes, these immersed in boiling water for 10 minutes, and then compared. The extreme amounts of Fehling solution used were 2 cc. and 11 cc. The end-point, or amount of action, was taken to be the last tube which failed to show any blue color. In a series of test-tubes ranging from those in which the yellow precipitate was so finely divided that it did not settle, through no blue color to a deep blue, the end-point could readily be obtained. Duplicates were run throughout.

In order to obtain the actions of a vegetable juice at different hydrogen ion concentrations, the following experiments were performed. (1) A preliminary series without incubation with 3 to 11 cc. of Fehling solution for the fresh juice and 0.50 to 5.00 cc. for the dehydrated vegetable juice in order to find the approximate end-points. (2) Blank and incubation experiments at hydrogen ion concentrations ranging from pH 2 to 11. The juice and the starch solution were brought to the requisite acidities and mixed. Sets of twelve tubes were used in each series of hydrogen ion concentrations and the amounts of Fehling solution varied progressively. (3) A series with the boiled juice at its natural hydrogen concentration. (4) A series with 0.25 cc. of juice plus 0.25 gm. of residue in place of the 0.50 cc. of juice at its natural hydrogen ion concentration.

The results were recorded in terms of cc. of Fehling solution reduced. No attempt was made to calculate the actions according to the Lintner scale. The actual amount of material in the juice was not known and varied considerably.

The Wohlgemuth^a method for amylase was found to be unsatisfactory for this work for the following reasons: (1) The difficulty of determining the end-point in some of the vegetable juices, such as the carrot and yellow turnip, which are colored. Instead of obtaining a series of clear colors from orange or yellow through purple to blue, greenish, greyish, and dirty brown mixtures were obtained. (2) The vegetable extracts themselves contained varying amounts of starch so that hydrolysis of the added starch

^a Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 1.

could not be taken as an indication of the activity unless correction was made for the starch present in the extracts. This correction was obviated in the Lintner method in which amylase action was measured by the total reducing products whether produced by the action of the amylase on the added starch or that naturally present in the extract. (3) In some of the vegetables, difficulty was caused by the presence of some chemically unsaturated or iodine-combining compound which united with the iodine and prevented the development of the blue color with the starch. It was found that 1 cc. of carrot juice combined with 2 drops of 0.1 N iodine solution in 5 minutes, 1 cc. of yellow turnip juice with 6 drops, 1 cc. of potato juice with 3 drops immediately, with 6 drops in 5 minutes, and with considerably more on longer standing, 1 cc. of cabbage juice with 4 drops immediately and more on standing.

Experimental Results.

	Percentage of total wt. as juice.	Specific gravity of juice.
Cabbage (fresh).....	45-60	1.028-1.031
“ (vacuum-dehydrated).....		1.025
“ (air-dehydrated).....		1.018
Carrots.....	25-30	1.040-1.044
“ (vacuum-dehydrated).....		1.020
“ (air-dehydrated).....		1.025
Yellow turnips.....	45	1.040-1.043
White “ (fresh).....	45-55	1.025-1.035
“ “ (vacuum-dehydrated).....		1.025
“ “ (air-dehydrated).....		1.025

Only with carrots was a marked difference apparent in the specific gravities of the juice from the fresh and the dehydrated products.

The hydrogen ion concentrations of all the vegetable juices, fresh as well as dehydrated, prepared as described, were found to be in the neighborhood of pH 6, with the exception of tomato juice which was very close to pH 4.

There was, however, a marked difference observed between the juices of fresh and of dehydrated products. In order to bring the hydrogen ion concentrations of the juices to various values

(between pH 2 and 12) normal acid and alkali were added. The amounts so added to a definite volume of juice to give definite hydrogen ion concentrations when plotted against these values (in terms of pH) give the titration curves of the substances whose forms probably depend upon the properties of the protein substances present. It was found in a comparison of the fresh and dehydrated vegetable juices that a change had occurred in the sense that less acid and more alkali were required to bring the juices of the dehydrated products to the same hydrogen ion concentrations than with the fresh. There was a greater change with the air-dehydrated than with vacuum-dehydrated products. For instance, with 100 cc. portions of the juices obtained from fresh, vacuum-dehydrated, and air-dehydrated white turnips, to bring them to pH 3 required 4.86 cc., 3.33 cc., and 2.17 cc. N HCl solution respectively, and to bring them to pH 9 required 1.43 cc., 2.67 cc., and 4.67 cc. N NaOH solution respectively. These changes were observed for a number of vegetables, but the results are not considered to be quantitative enough to warrant publication in detail. The qualitative observation must be emphasized, however, that there are definite changes brought about in dehydration as shown by the titration curve analyses, probably in the character of the protein material. In the paper by Cohn and his coworkers are given more quantitative data for potato and tomato juices and preparations showing conclusively that even very mild treatments influence the properties of the substances.

The results of the enzyme tests are given in Tables I to IV.

The enzyme reactions of all the vegetable juices studied were destroyed by heating to boiling for several minutes.

The oxidase reactions with carrot, yellow and white turnip, and potato juices were greatly increased on dilution. Evidently some substance (chemically unsaturated) was present which combined with the oxygen preventing it from acting on the reagent. The peroxidase reaction did not show this increased action on dilution. If oxidase and peroxidase are due to the same, or at least similar, grouping, then the addition of the hydrogen peroxide would account for the difference in behavior on dilution. The oxidase and peroxidase results are not quantitatively comparable since a much more sensitive reagent was used with the former.

There is no well defined hydrogen ion concentration for maximum action with oxidase, peroxidase, and catalase. In general terms, the actions were better in the more alkaline solutions, best on the average between pH 7 and 10. They were inhibited in acid solution, pH 2 and 3 for oxidase and peroxidase, and pH 4 for catalase, except in the case of the tomato. Inhibition occurred with these enzymes in some cases at pH 11 or 12.

With regard to the changes brought about by dehydration on the behavior of these three enzymes, the vacuum-dehydrated cabbage and carrot gave stronger oxidase reactions than did the

TABLE I.
Oxidase Tests on Vegetable Juices.

pH	Cabbage.								Carrot.				Yellow Turnip.			
	Fresh.		Fresh. 1:16		Vacuum-de- hydrated.		Vacuum-de- hydrated. 1:16		Fresh.		Fresh. 1:16		Fresh.		Fresh. 1:16	
	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.	↓ min.	15 min.
2	0	0			0	0	0	0			0	0	0	0	0	0
3	1-	1	1r*	1	1r	1	2-	2	0	0	1	1			1-	1-
4	1+	3r	1r	1	1-	1+	2-	2	1r	0	1	1+			1	1+
5	1+	3r			1	2	2-	2	2r	3r	1	1+	0	0	1	1+
6	1+	1+	1+	1+	1	2	2-	2	1	3r	2	2			1	1+
7	1+	2.			1	2	1-	2	1	3r	2	2			1	1+
8	2-	2			1	2	2-	2+	1	3r	2	2	0	0	1	1
9	0	0	0	0	1+	2	2-	2+	1	3r	2	2			1	1
10					1	2	1	2	1	2	2	2			1+	1+
11					0	0	1	2	0	1+	2	2	0	0	2	2
							↓ min.	15 min.				↓ min.				
	Juice (air-dehy- drated) pH 6.....								Juice (vacuum).....				0			
	Solid residue (fresh)								" (air).....				0			
	Solid residue (vac- uum).....								Solid (fresh).....				2			
	Solid residue (air).								" (vacuum).....				1			
									" (air).....				2			

* r indicates that the reaction was given on the surface where the reagent first came in contact with the liquid but disappeared on shaking or mixing the solution.

TABLE I—Concluded.

pH	White Turnip.								Potato.								Tomato.
	Fresh.		Fresh. 1:16		Vacuum-de- hydrated.		Vacuum-de- hydrated. 1:4		Fresh.		Fresh. 1:8		Vacuum-de- hydrated.		Fresh.		
	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	‡ min.	15 min.	
2	0	0							0	0	0	0			0	0	
3					0	0	1	1									
4			1	2	1	1	1	2	1	1	1	2			1	1	
5	0	0	2	2	1	2	1	2	1	1	1	2+	0	0			
6			1+	2	1	2	2	2	1	2	1+	2+	0	1			
7			1	2	1	2	2	2			1+	2+	0	1			
8	0	0	1+	2	1	2	2	2			1+	2+					
9			1	2	1	2	2	2			1+	2+					
10					1	2	2	2			1+	2+					
11	0	0	0	0	0	1	1	1			2	2+					
									‡ min.		15 min.				Juice (vacuum)		
	Juice (air) pH 6								0	0	Juice (air 40).....		0	0	0		
	Solid (vacuum)								1	1	" (air 60).....		1	1	Juice (air)		
	" (air)								0	2	Solid (fresh).....		1	1+	0		
											" (vacuum)...		0	2+	0		

fresh. In every other case the enzyme action was less in the juice from the dehydrated vegetable than in that from the fresh. With regard to the materials prepared by the two methods of dehydration, vacuum and air blast, the latter gave substances in which the enzymes were destroyed more completely than the former. It is possible that part of this inhibition or destruction of enzymic property was due to changes in the material resulting in differences of extraction. However, against this view is the fact that enzyme tests on the solid residues from the extractions also showed that air dehydration caused greater destruction of the enzymes than did vacuum dehydration.

Oxidase could not be studied in tomato and potato juices at hydrogen ion concentrations less than those indicated because of the interference by the color of the juices.

TABLE II.
Peroxidase Tests on Vegetable Juices.

pH	Cabbage.								Carrot.				Yellow Turnip.			
	Fresh.		Fresh. 1:16		Vacuum-de- hydrated.		Vacuum-de- hydrated. 1:16		Fresh.		Fresh. 1:16		Fresh.		Fresh. 1:16	
	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.
2	0	0			0	0										
3	2	3	1-	1+	1+	2-	0	0					0	0	0	0
4	2	3	1-	1	1+	2-	0	1-	1	2	0	0	1	2	1-	2-
5	2+	3			1+	2	0	1-	1	2			2+	3+	1-	2
6	2+	3	1-	1+	1+	2	0	0	1+	2+			2+	3+	1-	3
7	2+	3			2-	2+			1+	3	0	0	2+	3+	2-	3+
8	3	3+			2	3			1+	3			2+	3+	2-	3+
9	3	3+	1-	2+	3-	3			1+	3			2+	3+	2	3+
10	2	3+			2+	3			1+	3	0	0	2+	3+	2+	3+
11	2	3+	1-	2+	0	2-			1	2			3	3+	2+	3
Juice (air), pH 2-11 0								Juice (air).... 0 Solid (fresh).. 2+ " (vacuum) 2 Solid (air).... 0								
White Turnip.								Potato.				Tomato.				
pH	Fresh.		Fresh. 1:16		Vacuum-de- hydrated.		Vacuum-de- hydrated. 1:16		Fresh.		Fresh. 1:8		Fresh.			
	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.	↑ min.	30 min.		
2							0	0	0	0			0	0		
3	0	0			0	0	0	1-	0	1			1	2		
4	2	3	0	1	1+	2	0	1-	1	1+	0	0	1	2		
5	2+	3+			1+	2	0	1	2	2+	1-	1	1+	2+		
6	2+	3+	0	2	1+	3	0	1+	2	3+	1-	1+	2	3		
7	2+	3+	0	2+	2-	3	0	1+	3	3+	1-	1+	2	3		
8	2+	3+			2	3	0	1+	3	3	2	2	2	3		
9	2+	3+	0	3-	3	3	1	2			2	2	1+	2+		
10	2+	3+	0	3-	1	2+	1	1+			2-	2	1+	2+		
11	1	3	0	0	0	0	0	0			2-	2	1	1+		
Juice (air)..... 0 (pH 6) Solid (fresh).... 3 " (vacuum) . 3 " (air)..... 3								Juice (vacuum)..... 0 Juice (air)... 0 Solid (vacuum)..... 2+ Solid (air).. 0				Juice (vacuum).... 0 (pH4) Juice (air) 0 (pH4)				

TABLE III.

Catalase Tests on Vegetable Juices.

TABLE III—*Concluded.*

pH	White Turnip.								Tomato.	
	Fresh.		Fresh. 1:16		Vacuum-de- hydrated.		Vacuum-de- hydrated. 1:4		Fresh.	
	† min.	30 min.	† min.	30 min.	† min.	30 min.	† min.	30 min.	† min.	30 min.
2									1+	2
3									1	2
4	0	0	0	0					2	2+
5	0	1			0	0	0	0	2	2+
6	1+	2	1-	1-	1	1	0	1-	3	3
7	2+	3+	1-	1-	1	1	0	1-	3	3
8	2+	3+			1	1	1-	1-	3	3
9	2+	3+	1-	1-	1	1	1-	1-	3	3
10	2+	3	1	1	1	1	1-	1-	3	3
11	1+	2	1-	1-	1-	1	1-	1-	2-	2-
					† min.	30 min.				
	Juice (air)...				0	0			Juice (vacuum).....	0
	Solid resi- due (vac- uum).....				1	2+			" (air).....	0
	Solid resi- due (air)...				1	1-			Solid (fresh).....	1
									" (vacuum).....	1
									" (air).....	1

These optimum actions are of interest in comparison with the optimum actions described by Sherman, Thomas, and Baldwin,⁹ pH 7 for pancreatic amylase, pH 4.4 to 4.5 for malt amylase, and pH 4.8 for *Aspergillus oryzae* amylase. The optimum hydrogen ion concentrations for the vegetable juice amylases coincides with the natural hydrogen ion concentrations of these juices. Testing mixtures of the extract and the solid residue of the cabbage and yellow and white turnip gave results which indicated that two-thirds or more of the total activity was contained in the juice as prepared.

A comparison of the values of the blanks and the maximum actions shows that the larger the blank the greater the action.

⁹ Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.

In other words, the greater the amount of amylase in the juice, the greater the amount of reducing substance in the vegetable, or the greater the action of the amylase in the vegetable itself. Potato juice, when tested for amylase by the method used with the other vegetable juices, gave an end-point which was so uncertain and difficult to determine that a different method had to be employed for it. These results will be communicated in a later paper.

TABLE IV.

Amylase Tests on Vegetable Juices.

	pH	Cabbage.			Carrot.		Yellow Turnip.	White Turnip.	
		Fresh.	Vacuum-dehydrated.	Air-dehydrated.	Fresh.	Vacuum-dehydrated.	Fresh.	Fresh.	Vacuum-dehydrated.
	3	0	0		0		0.20	0.30	0.15
	4	1.65	0.50		0.25		2.70	1.30	0.65
	5	2.15	1.00		0.25		2.45	1.55	0.65
	6	2.40	1.25	0.80	0.60	0.15	2.30	2.40	0.65
	7	1.90			0.25		2.70	1.30	0.15
	8	0.90	0		0.25		0.70	0.80	
	9	0.40			0		0	0.20	
	10	0						0	
Blanks (mean)		4.60	3.00	1.20	2.50	1.60	7.30	3.70	3.35
Maximum actions.....		2.40	1.25	0.80	0.60	0.15	2.70	2.40	0.65

The effect of dehydration on the amylase content is the same as with the other enzymes, decreased action in every case, but greater decreased actions with air dehydration than with vacuum dehydration.

A number of preliminary experiments were carried out with the vegetable juices in the presence of different salts in which the Wohlgemuth method was used. Little difference was found in the amylase actions with the added salts, so that it may be assumed that the concentrations of the electrolytes in the natural juices were sufficient to give very nearly optimum conditions.

CONCLUSIONS AND SUMMARY.

The properties of the oxidase, peroxidase, catalase, and amylase of cabbage, carrot, yellow and white turnip, tomato, and potato juices were studied at different hydrogen ion concentrations. Marked changes in the enzyme contents and properties were found on dehydrating these vegetables, air blast dehydration producing considerably greater changes than vacuum dehydration.

In considering these enzyme results, it may be pointed out that the state of ripeness and the age of the vegetable undoubtedly influence the activities. More complete series of results taking these factors into account would give results of interest. In this work, ordinary fresh vegetables, as purchased in the open market, were used.

With regard to a practical question of dehydration, the air-dehydrated potatoes prepared commercially are boiled before dehydration. Those used in this investigation were not boiled. The vacuum-dehydrated potatoes were obtained white in color, the air-dehydrated grey to black. On treating the vacuum-dehydrated potatoes with water of the hydrogen ion concentration 10^{-5} N or more, no darkening was noticeable, with water of the hydrogen ion concentration 10^{-6} N or less, comparatively rapid blackening occurred. The potato in all probability has a natural hydrogen ion concentration of 10^{-6} to 10^{-7} N.¹⁰ If this could be increased to 10^{-5} N before dehydration, then even air dehydration should give a colorless product. Treatment with dilute acid solution before dehydration may accomplish this.

Since heat and time are probably the determining factors in bringing about the changes in enzyme actions, it would appear that the shorter the period of time in which heat is applied, the smaller will be the changes. Vacuum dehydration can be carried out in 8 to 12 hours or less; air blast dehydration requires longer periods of time. This may be one of the reasons, in addition to the possible changes brought about by the oxygen of the air, for the smaller changes in the vacuum dehydration products as compared with the air blast dehydration products.

¹⁰ Cohn, E. J., and Gross, J. Data to be published later in *J. Gen. Physiol.*

This point may also be mentioned in connection with the food hormones. It is possible that cooking at a high temperature for a short period of time¹¹ will not cause the destruction of these properties, that heating in a current of air for a long time will destroy them, and that heating for an intermediate period of time in the absence of air may do so partially.

The differences in the titration curves between the juices from the fresh and the dehydrated vegetables indicate also that some change takes place on dehydration, greater in air blast dehydration than in vacuum dehydration.

With regard to the relation between the chemical properties or structures upon which the actions of enzymes and of food hormones depend, one significant fact is that enzymes are inactivated by heating in solution for a short time, while food hormones as a rule are not. On the other hand, the partial or complete inactivation of both in many cases in dehydration indicates a possible similarity. No evidence is available as to the nature of the chemical groupings responsible for these actions. All that can be said at present is that the enzyme property is destroyed more readily than the food hormone properties. The enzyme property is perhaps the most sensitive index of change taking place in material obtained from living matter susceptible to tests by simple chemical means. A change in the enzyme properties need not be accompanied necessarily by a change in the properties of the food hormones.

¹¹ Delf, E. M., *Biochem. J.*, 1918, xii, 416.

ACIDIMETRIC TITRATION OF GRAIN EXTRACTS AND AMINO-ACIDS IN THE PRESENCE OF ALCOHOL.*

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The observation described here was first made in December, 1915, during a study of the sugar content of several aqueous extracts of oats. A number of finely ground samples of oats had been extracted with ice water in a cold storage room for 1 hour and the filtered extracts were neutralized with 0.1 N sodium hydroxide, phenolphthalein being used as an indicator. A large excess of neutral ethyl alcohol was then added in order to precipitate dextrans and other gummy substances. I noticed that if alcohol was added to a given aliquot of the cold extract before titration considerably more alkali was required for complete neutralization than when no alcohol was present. This apparent change in acidity took place immediately after adding the alcohol, notwithstanding the low temperature of the two liquids and of their mixture. Extracts of other cereals behaved in the same way. In most cases a precipitate was formed upon the addition of alcohol. When the aqueous extract to which alcohol had been added was heated without previous neutralization until all the alcohol was driven off, the precipitate did not redissolve upon the addition of distilled water, but the acidity of the resulting solution was the same as that of the original aqueous extract. This shows that the substances which are precipitated or coagulated by the alcohol probably bear no direct relation to the shift in acidity.

It seemed important to investigate the influence which different concentrations of alcohol might have on the titration value

*An abstract of this paper was read before the 33rd annual convention of the Association of Official Agricultural Chemists at Washington, D. C., in November, 1916.

of the mixture. I found for oats that the titration values do not increase in linear proportion to the amount of alcohol which was present, but that the rise is slow in the beginning, then gradually becomes more rapid, and finally the values remain constant. If plotted on paper, they form an S-shaped curve (Fig. 1).

If mixtures of oats or corn extracts with ethyl alcohol were allowed to stand for several days at either ordinary or cold storage temperatures, without being neutralized, their acidity remained practically unchanged; if neutralized, more acid was formed. Moreover, a greater amount of acid was produced in cases when the mixtures had been neutralized at once than when they had stood for several days before being neutralized. Possibly the formation of fresh acid is due to an enzyme which is destroyed during prolonged contact with alcohol. It may be assumed that the enzyme possesses acid properties. This would explain the fact that in the presence of alcohol the acidity values of unneutralized extracts are a trifle lower after several days standing than at the start (see Tables II and III).

Experiment 1.—18 gm. of ground oats were extracted with 150 cc. of ice water for 70 minutes. Of the filtered extract, 25 cc. aliquots were pipetted into two Jena beakers. The one aliquot was neutralized with 0.1 N NaOH, with phenolphthalein as indicator. 0.68 cc. was required to produce a pink color. To both aliquots 35 cc. of neutral absolute alcohol were now added. A white precipitate was formed in each one, and in the aliquot which had already been neutralized the pink color disappeared. An additional 0.82 cc. of 0.1 N alkali was required to produce again the pink coloration. The amount of alkali required to neutralize the 25 cc. of aqueous oat extract to which 35 cc. of neutral alcohol had been added was, therefore, more than twice the amount required when no alcohol was present.

The other beaker, the contents of which had not been neutralized, was placed on the steam bath until all the alcohol had been driven off. Care had to be exercised not to carry the evaporation too far, since otherwise decomposition takes place, which is accompanied by a rise in acidity. After adding about 20 cc. of distilled water, the liquid required 0.69 cc. of 0.1 N NaOH for neutralization. The white precipitate had not redissolved.

With 18 gm. of corn-meal, if extracted in exactly the same manner, similar results were obtained, the values found being as follows:

25 cc. of extract required directly.....	0.87 cc. of 0.1 N NaOH
With 35 cc. of alcohol added there were	
required an additional.....	0.73 " " 0.1 N NaOH
Total.....	1.60 " " 0.1 N NaOH

The evaporated aliquot, after adding
water, required..... 0.86 cc. of 0.1 N NaOH

Experiment 2.—Measured amounts of neutral absolute alcohol were placed in dry 100 cc. Erlenmeyer flasks and the latter closed with rubber stoppers. 156 gm. of ground oats (containing 5.8 per cent of moisture) were extracted with 1,200 cc. of ice water for 70 minutes, with occasional stirring. The infusion was poured upon folded filters, and the filtrates were united and well mixed. 20 cc. portions of this filtrate were measured from a burette into each of the Erlenmeyer flasks containing different amounts of alcohol. Each mixture was made up in duplicate. The extraction of the oats and the measuring off of the 20 cc. aliquots were carried out in a cold storage compartment having a temperature of 1–2°C. The various alcohol-extract mixtures were then titrated with 0.1 N NaOH, phenolphthalein being used as indicator.¹ The titration values are given in Table I.

In Fig. 1 are shown the S-shaped curves which represent the changes in acidity with varying concentrations of alcohol. In the lower curve the strength of the alcohol is expressed in volumetric per cent, while in the upper the abscissæ represent the number of cc. of alcohol which in each case was mixed with 20 cc. of extract. The curves seem to show a second short rise between the alcohol concentrations of 60 and 65 per cent; that is, just before reaching the final maximum. The experiment was repeated twice and this second rise was observed in each case.

Experiment 3.—To 20 cc. aliquots of ice water extracts of oats and corn-meal were added measured amounts of neutral alcohol. The mixtures were made up in triplicate. The one set was neutralized at once, the

¹ McCoy (*Am. Chem. J.*, 1904, xxxi, 508) noted that the addition of alcohol reduces the color intensity of alkaline solutions of phenolphthalein. This phenomenon, for which he offered no satisfactory explanation, is doubtless responsible to a slight extent for the rise in acidity reported in this paper. However, since not more than five drops of a 0.5 per cent solution of phenolphthalein (previously neutralized to pale red) were used for any of my titrations, the shift due to the indicator may be considered as negligible. Morey (*J. Am. Chem. Soc.*, 1912, xxxiv, 1031), in titrating benzoic acid in strong alcohol with three drops of 1 per cent phenolphthalein as indicator, required only from 0.06 to 0.08 cc. of 0.1 N alkali in his blanks. I have myself titrated a number of weak acid solutions, *e.g.* of acetic, tartaric, and lactic acid, in the same manner as the grain extracts without obtaining an appreciable shift of acidity in the presence of alcohol.

TABLE I.

Relation Between Concentration of Alcohol and Neutrality Point (Phenolphthalein) of an Ice Water Extract of Oats.

Alcohol added to 20 cc. of extract.	Alcohol content of mixture.	0.1 N NaOH required.	Average.
cc.	per cent	cc.	cc.
0	0	0.60 0.60	0.60
1	4.8	0.62 0.62	0.62
3	13.0	0.66 0.66	0.66
5	20.0	0.71 0.73	0.72
7	25.9	0.83 0.83	0.83
9	31.0	0.94 0.95	0.945
11	35.5	1.05 1.05	1.05
13	39.5	1.11 1.13	1.12
15	42.9	1.18 1.20	1.19
17	46.0	1.23 1.25	1.24
19	48.7	1.28 1.30	1.29
21	51.2	1.32 1.32	1.32
23	53.5	1.34 1.36	1.35

TABLE 1—*Concluded.*

Alcohol added to 20 cc. of extract.	Alcohol content of mixture.	0.1 N NaOH required.	Average.
cc.	per cent	cc.	cc.
30	60.0	1.39 1.41	1.40
32	61.5	1.41 1.41	1.41
34	63.0	1.43 1.47	1.45
36	64.3	1.44 1.46	1.45
38	65.5	1.47 1.47	1.47
40	66.7	1.47 1.45	1.46
42	67.7	1.47 1.48	1.475

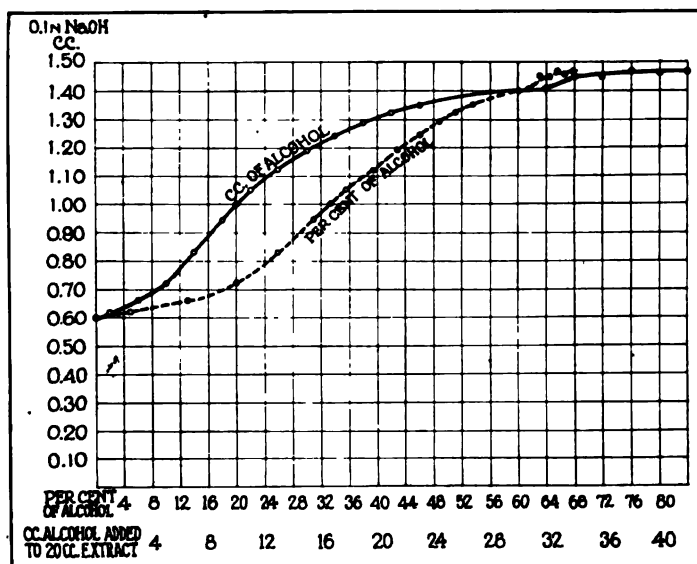


FIG. 1. Relation between concentration of alcohol and neutrality point (phenolphthalein) of an ice water extract of oats.

TABLE II.

Effect of Keeping Alcohol-Extract Mixtures for Some Time before and after Neutralizing.

Alcohol added to 20 cc. of extract.	Titrated at once.		Titrated after 24 hrs. at 1°C.		Titrated after 48 hrs. at 1°C.		Titrated after 48 hrs. at 25°C.	
	Original titration.	Increment 2 days later.	Original titration.	Increment 2 days later.	Original titration.	Increment 2 days later.	Original titration.	Increment 1 day later.
	0.1 N NaOH required for neutralisation.							
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Oats.								
0	0.69	0.20	0.69	0.20				
5	0.87	0.24	0.82	0.17				
10	1.12	0.40	1.07	0.11			1.15	0.09
15	1.28	0.45	1.29	0.11			1.28	0.11
20	1.42	0.65	1.37	0.13			1.34	0.10
25	1.48	0.61	1.41	0.24			1.38	0.12
Corn-meal.								
0	0.88	0.25			0.88	0.25		
5	1.09	0.18			1.05	0.11		
10	1.30	0.22			1.20	0.13	1.20	0.13
15	1.46	0.23			1.30	0.18	1.33	0.11
20	1.55	0.31			1.39	0.21	1.40	0.15
25	1.65	0.32			1.44	0.31	1.50	0.12

With another oat extract, the results were as follows:

TABLE III.

Effect of Keeping Alcohol-Extract Mixtures before Neutralizing.

Alcohol added to 30 cc. of oat extract.	0.1 N NaOH required for neutralisation.	
	Titrated at once.	Titrated after 72 hrs. at 1°C.
	cc.	cc.
12	1.15	1.10
16	1.21	1.23
20	1.31	1.30
25	1.35	1.34
30	1.39	1.35
35	1.47	1.43

second was placed in the cold storage room in stoppered flasks before being neutralized. The third set, containing only four mixtures, was kept at room temperature. The time during which the different mixtures were kept, before being neutralized, is indicated in Tables II and III.

It is seen from Tables II and III that the acidity of these extracts to which alcohol had been added does not change markedly on standing, and that whatever slight changes may take place consist in a destruction of acid substances.

After the first neutralization, the various mixtures of alcohol and oat extract formed more acid. The amounts of this secondary acid formation were determined by a new titration after standing. They are designated as increments in Table II. It is seen that if the first neutralization has taken place soon after adding the alcohol to the extract, the secondary acid formation was much stronger than if the liquids had not been neutralized at once, a fact which may be due to a gradual disintegration of acid-forming ferments.

Experiment 4.—Samples of 15 gm. each of the following ground materials were extracted with 150 cc. of ice water for 70 minutes: polished rice, pearl barley, whole wheat, whole rye, white kidney bean. The extracts were filtered and duplicate aliquots of 30 cc. each (corresponding to 3 gm. of material) pipetted into 100 cc. Erlenmeyer flasks. To the one aliquot of each, 30 cc. of neutral absolute alcohol were added, while the other aliquot was titrated directly, using phenolphthalein as indicator. The results are shown in Table IV.

TABLE IV.
Shift in Acidity of Ice Water Extractions of Various Seeds Caused by Addition of Alcohol.

	0.1 N NaOH neutralising 30 cc. of extract (= 3.0 gm. of substance).	
	Without alcohol.	After adding 30 cc. of neutral alcohol.
	cc.	cc.
Polished rice.....	0.12	0.25
Pearl barley.....	0.71	1.01
Whole wheat.....	0.46	1.07
“ rye.....	1.57	2.88
White kidney bean.....	3.10	5.68

It is seen from Table IV that the phenomenon reported above is not limited to cereals but that it also occurs with extracts of other seeds. However, no secondary acid formation took place in the case of the bean extract, while all grain extracts, after being neutralized, formed fresh acid if allowed to stand for a few hours.

Inasmuch as the facts observed seemed to have some bearing on the interpretation of titration results which have been sometimes obtained in the presence of alcohol,² a brief attempt was made to determine the nature of those grain constituents which are responsible for this remarkable shift in acidity. It was soon found that the phenomenon is probably attributable to amino compounds present in the extracts, since, as demonstrated in the following experiments, solutions of these substances show the same behavior.

Dilute aqueous solutions (approximately 0.12 per cent) of tyrosine and leucine were prepared. To 25 cc. aliquots of these solutions 25 cc. of neutral alcohol were added, and in a control experiment 25 cc. of distilled water. On neutralizing these liquids, with phenolphthalein as indicator, the following results were obtained:

	0.1 N NaOH required.	
	Without alcohol.	In 50 per cent alcohol.
	cc.	cc.
Leucine.....	0.08	2.24
Tyrosine.....	0.44	2.50

Therefore, with these two monoamino-acids, a strong shift in acidity actually took place upon adding ethyl alcohol.

The matter was further studied with solutions of glycocoll, alanine, aspartic acid, and hippuric acid, all of Kahlbaum's manufacture. The results are set forth in Table V, and graphically in Fig. 2.

² A paper on this subject by myself will appear shortly in the *Journal of Agricultural Research*. The recent findings of Ferraro (*Boll. chim. farm.*, 1915, liv, 257, abstracted in *Chem. Abstr.*, 1916, x, 3042) harmonize with my own observations.

TABLE V.

Change in Acidity of Amino-Acid Solutions, Caused by Addition of Alcohol.

Alcohol added before titration to 10 cc. of aqueous solution of acid.	0.05 N NaOH required to produce pink coloration of phenolphthalein.			
	Glycocoll.	Alanine.	Aspartic acid.	Hippuric acid.
cc.	cc.	cc.	cc.	cc.
0.0	0.40	0.18	2.50	1.41
1.5	0.55			
2.0		0.50		
2.5	0.85			
3.0				1.41
4.0		1.30		
5.0	2.00		2.85	
6.0		1.63		1.41
7.5	3.00			
8.0		2.38		
10.0	3.70	2.90	3.35	1.41
12.5	3.90	3.30		
15.0	4.40	3.60	3.65	1.41
17.5	4.40	4.00		
20.0		4.20	3.80	
25.0		4.45	3.85	

The solutions of the two monoamino-acids were of approximately the same molecular strength, containing per 100 cc. respectively, 0.198 gm. of glycocoll, and 0.235 gm. of alanine. The acidity of the latter is seen to rise more slowly than that of the former, but both appear to reach the same maximum.

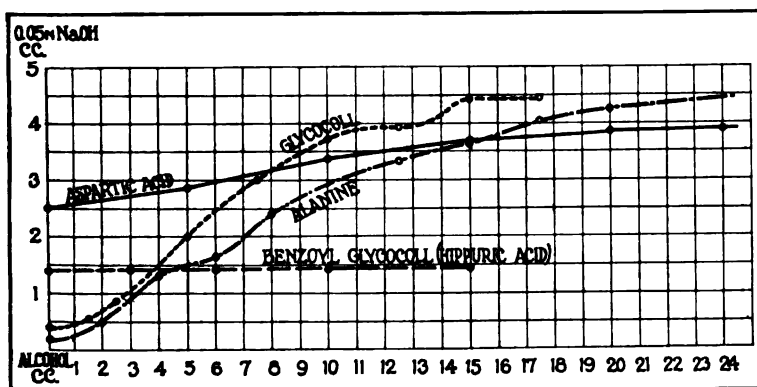


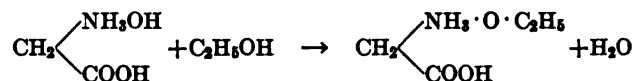
FIG. 2.

With the dibasic aspartic acid, of which an 0.18 per cent solution was used, the increases due to alcohol, although quite measurable, are much less pronounced than with the monobasic acids.

Unfortunately arginine or histidine could not be tested for want of material. These two amino-acids, which in aqueous solution possess an alkaline reaction, may be expected to react acid to phenolphthalein in the presence of sufficient alcohol.

Hippuric acid, which is not an amino-acid, but which may be obtained synthetically from glycocoll, was included in this study merely to show that the phenomenon in question is very likely confined to compounds containing the NH_2 group. The solution used contained 0.125 gm. of hippuric acid per 100 cc. No rise in acidity was observed upon addition of alcohol.

The phenomenon described is possibly due to a simple substitution reaction with the hydrated form in which, according to Robertson,³ amino-acids exist in aqueous solution, and to which he attributes their amphoteric character. This reaction could be expressed in the case of glycocoll, by the equation



and the elimination of the hydroxyl group would account for the loss of the amphoteric character of these bodies in the presence of alcohol.

The observation may have practical importance, inasmuch as under certain conditions it may furnish a convenient measure for the rate of decomposition of the proteins contained in a given solution.

SUMMARY.

The main result of this study may be stated in the following terms.

Amino-acids, which in aqueous solution are nearly neutral to phenolphthalein, react distinctly acid in the presence of alcohol. This fact should be taken into account when making acidimetric titrations in alcoholic liquids containing amino compounds, such as various animal or vegetable extracts.

³ Robertson, T. B., *Ergebn. Physiol.*, 1910, x, 216.

PREPARATION OF RHAMNOSE.

By E. P. CLARK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 29, 1919.)

Methods for the preparation of rhamnose were published by Kruis¹ and by Rayman.² These authors found that commercial extract of black oak bark known as "quercitron extract" was a convenient material for the preparation of this sugar. The process employed by these authors was as follows.

Quercitron extract was hydrolyzed by boiling with dilute sulfuric acid for $\frac{1}{2}$ hour. This solution was filtered and neutralized with calcium or barium carbonate. The solution after filtering was concentrated to a thick syrup and allowed to crystallize.

When this process was applied to material obtainable at the present time the result was very unsatisfactory. However it was found possible to obtain quite satisfactory yields of rhamnose by a modification of the original method. The modified process is as follows.

2 kilos of the liquid quercitron extract,³ which has the consistency of a thick syrup, is dissolved in enough hot water to make $5\frac{1}{2}$ liters. It is brought to a boil and 180 cc. of concentrated sulfuric acid dissolved in 320 cc. of water are gradually added, thus making the concentration of the acid 3 per cent by volume. The mixture is gently boiled for 30 minutes, then poured into a crock jar or enameled kettle, and quickly cooled.

The liquid after being separated from the insoluble material is neutralized with barium hydroxide. The barium sulfate is filtered off, the filtrate evaporated to a thin syrup (approx-

¹ Kruis, K., *Sitzungsberichte der Böhmischen Gesellschaft der Wissenschaften*, Prague, 1877-78, 157.

² Rayman, B., *Bull. Soc. Chim.*, 1887, xlvii, 668.

³ The material used in this work was obtained from A. Klipstein and Co. of New York.

mately 350 cc.), and 8 volumes of 95 per cent alcohol are slowly added with constant stirring. The heavy precipitate formed is filtered off and sucked dry on a Büchner funnel. The resulting filtrate is evaporated under diminished pressure to a thick syrup, dissolved in 1 liter of 95 per cent alcohol, and to the solution $2\frac{1}{2}$ liters of ether are added. A gummy substance is precipitated, which, after decantation of the supernatant liquid, is dissolved in 50 cc. of water and enough alcohol to make 1 liter, the alcohol being slowly added and thoroughly mixed with the syrup. The solution is precipitated again with $2\frac{1}{2}$ liters of ether. The liquid is decanted from the precipitate and the two ethereal extracts mixed and allowed to stand over night during which time they become clear. The clear solution is poured off from the gummy substance which settled out, the ether is recovered by distilling on a steam bath, and the remaining alcoholic solution is evaporated under diminished pressure to a thick syrup. This is removed from the flask with an equal volume of alcohol. To the solution ether is then added. Upon scratching the flask, or quicker by seeding, the rhamnose separates out, requiring generally a day for complete crystallization. The sugar is filtered off as dry as possible on a Büchner funnel, washed first with a mixture of 1 part ether and 2 parts alcohol, then with a 1:1 alcohol and ether mixture, and finally with ether.

The yield is from 50 to 55 gm. of white sugar. It may be recrystallized from water or alcohol in the usual way.

THE STABILITY OF LACTALBUMIN TOWARD HEAT.

By A. D. EMMETT AND G. O. LUROS.

(From the Research Laboratory, Parke, Davis and Company, Detroit.)

(Received for publication, April 21, 1919.)

Since there seems to be some specific and characteristic difference between the growth-promoting value of casein and lactalbumin, which is dependent upon certain dietary conditions as shown by comparing the results of Osborne and Mendel,¹ McCollum, Simmonds, and Parsons,² and Emmett and Luros,³ we have made a further study of this subject by undertaking to ascertain whether heat will affect the nutritive value of lactalbumin to the extent that McCollum and Davis claim that it destroys the growth-promoting value of casein.

McCollum and Davis⁴ reported that, by long continued heating of milk at 90-100°, the nutritive value of the casein was impaired; that, when casein was heated in an autoclave for 1 hour at 15 pounds pressure, it lost its "biological value as a complete protein," due to some of the amino-acids being broken down; that wet milk powder heated for a time in a double boiler or for 1 hour in the autoclave at 15 pounds pressure was deficient in promoting growth; that evaporated milk whey was not affected by heating in the autoclave; and that unrefined lactose retained as much of the adhering water-soluble B accessory after heating as before. In view of these findings, McCollum and Davis concluded that it would not be practicable to purify casein by washing it with hot alcohol as Funk and Macallum⁵ had done, and so proposed a method of purification which did away with the use of heat entirely. Funk and Macallum in turn prepared casein by these two methods and found that there was no difference be-

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351; *ibid.*, 1916, xxvi, 1.

² McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.

³ Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 147.

⁴ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231, 247.

⁵ Funk, C., and Macallum, A. B., *Z. physiol. Chem.*, 1914, xcii, 17; *J. Biol. Chem.*, 1916, xxvii, 51.

tween them in respect to their growth-promoting value. Upon heating the casein in the autoclave, however, they obtained evidence which confirmed McCollum and Davis as to a loss in the biological value of casein. They attributed the change in the nutritive value of the protein to a destruction of the antiscorbutic vitamine, and not to a breaking down of the protein. Hogan² studied the effect of heat on the growth-promoting value of corn, egg albumin, and casein. His data indicate that when corn was heated in the autoclave, no growth resulted, while excellent results were obtained with unheated corn. Egg albumin, heated in the autoclave, and then combined with protein-free milk, butter, starch, and agar so as to make a 9 per cent protein diet, was very slightly, if at all, impaired. Upon heating casein in an autoclave under 15, 30, and 45 pounds pressure, respectively, for 2 hours, and making up three corresponding rations with protein-free milk, butter fat, starch, and agar, and comparing each with a ration containing unheated casein, Hogan obtained results which indicated that casein was not affected by heat. His conclusions were that heat does not lower the nutritive value of the casein, but that one or more of the food accessories may be affected.

In the series of tests reported here on the effect of heat upon the nutritive value of lactalbumin, we subjected this protein to different temperatures and then incorporated it in the basal diet. The control supply of lactalbumin was dried *in vacuo* at 55-60° (Chart 1). Portions of this lot were then heated as follows: (a) in the air oven at 90-100° for 16 to 18 hours (Chart 2); (b) in the autoclave at 15 pounds pressure for 2 hours (Charts 3, 4, and 5); and (c) in the autoclave at 15 pounds pressure for 6 hours (Chart 6). The basal diet was composed of protein-free milk 28 per cent; butter fat 5, 18, or 28 per cent; lard none or 10 per cent; and starch to make up the balance after adding the protein. In each case, 10 per cent of lactalbumin protein was employed, this being based upon the nitrogen content of each of the proteins.

The protocols accompanying the charts give the details of the different tests. It will be seen, from the curves showing the rate of growth for the different groups of rats, that heating the lactalbumin had no effect upon its growth-promoting value.

In view of this fact, it is of interest to compare these results with our former conclusions, that lactalbumin is a complete protein for growth,³ and to correlate the evidence that we put

² Hogan, A. G., *J. Biol. Chem.*, 1916, xxvii, 193; *ibid.*, 1917, xxx, 115

forth to show that the difference between lactalbumin and casein may be based on the vitamine hypothesis.

Studying the basal diets of McCollum and Davis, Funk and Macallum, and Hogan, it will be seen that their experimental rations differed in two essential points: first, in respect to the amount of butter fat used, and second, in respect to the source of water-soluble vitamins employed. Thus, McCollum and Davis used 5 per cent butter fat and obtained their water-soluble vitamins from powdered milk, milk whey, wheat germ, or lactose. Funk and Macallum employed no butter fat but obtained the fat-soluble A accessory from dried yeast which also furnished the water-soluble B. Orange juice was also used. Hogan incorporated 30 per cent of butter fat in his rations, and for the water-soluble vitamins he used either corn or protein-free milk.

From the standpoint of the amount of butter fat, it might be said that Hogan obtained normal growth with heated casein because he used 30 per cent of butter fat, while McCollum and Davis secured poor results on account of having only 5 per cent of the fat present. It is easy to conceive that the larger amount of butter fat might have altered the palatability of the ration so that the food intake was modified and thereby produced a difference in gain in weight; or it might be that this diet was more easily assimilated. Osborne and Mendel⁷ and Maignon⁸ found that the availability of protein varied directly with the amount of fat present. The data given in Charts 3, 4, and 5, where 18, 5, and 28 per cent, respectively, of butter fat was mixed with the 2 hour autoclaved lactalbumin, indicate clearly that the amount of butter fat had no effect upon the rate of growth. One group of rats grew as well as another and the body condition of each was equally good. This is brought out more clearly perhaps when the food intake is considered. Table I shows that the group consuming the low fat diet ate more food than did the one on the high fat ration. In other words, the low fat group consumed much more protein, and if the heated lactalbumin had been toxic, we would not have obtained results which were equal to those made by the high fat group where less protein was ingested. Further, in comparing the food intake for the heated and un-

⁷ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.

⁸ Maignon, F., *Compt. rend. Acad.*, 1918, clxvii, 172.

heated lactalbumin rations with 18 per cent butter fat and 10 per cent lard, the differences were so slight that it is evident that the heated protein was not toxic. These findings suggest that the percentage of butter fat in the ration had no special function in making the heated lactalbumin more available than the unheated protein, and it is more than likely that it had no influence with the heated casein which Hogan used.

With regard to the water-soluble vitamins, in the case of McCollum and Davis' report, it so happened that in their various rations which contained milk or its components, they had present, when they obtained good growth, some constituents of the milk which had *not* been heated. Thus, with the heated casein they had unheated milk whey or lactose while with the unheated

TABLE I.
Average Values for Each Group for Five Weeks.

Lactalbumin.	Fat.			Gain per day.	Food intake per day.	Gain per gm. of food.
	Butter fat.	Lard.	Total.			
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Unheated.....	18	10	28	1.56	6.6	23.6
6 hour autoclave.....	18	10	28	1.50	7.8	19.2
2 " ".....	18	10	28	1.34	6.5	20.9
2 " ".....	28	—	28	1.72	7.7	22.8
2 " ".....	5	—	5	1.60	12.9	12.4

casein they used heated whey or lactose. In other words, their combination of milk food substances was such that there was a good possibility that the unheated constituent carried an accessory (which we have already suggested as being able to be adsorbed by lactose and possibly by casein) and that this was responsible for the growth on the rations. To be sure the authors cover this point in part by showing that the wheat embryo can be heated for 1 hour in the autoclave at 15 pounds pressure and then when it is added to a diet of polished rice and butter fat, it is still able to supply the water-soluble B vitamin. However, from some of our results which are now in preparation for publication, we have evidence to show that heat will partially destroy a particular vitamin (other than the water-soluble B) that relates to growth, and that the rate of growth will depend largely

upon the amount of the substance present and also upon the degree of heating. That is, if the minimum amount or slightly more of the unheated material, which carries the vitamine, is present, normal growth will occur, but heating this vitamine will cause poor growth or no growth at all. If, however, there is an excess of the vitamine present in the unheated food, the effect of heating it will not be so marked.

Funk and Macallum obtained poor growth with heated casein even when dried yeast was in the ration, but when 1 cc. of orange juice was added each day they obtained good results. This stimulation of growth was attributed to the antiscorbutic vitamine in the juice. Hogan supplemented each of his casein rations with protein-free milk just as we did with lactalbumin. Protein-free milk contains 80 per cent of lactose and we⁸ have shown that lactose has some peculiar biological property of stimulating growth which is due either to its ability to overcome toxicity or else to a vitamine (other than the water-soluble B) which it appears to adsorb. Therefore, it may be that the lactose in the protein-free milk carried enough of the accessory or vitamine to effect normal growth with casein in Hogan's experiments. Chart 7 illustrates how the addition of lactose, as a partial substitute for starch in an otherwise complete diet, stimulated growth.

Our inference would be, therefore, that this accessory or vitamine was the factor that brought about the difference between the values of heated casein of McCollum and Davis on the one hand, and of Hogan on the other. This vitamine is different from the so called water-soluble B which is stable toward heat. Whether it is the same as the antiscorbutic vitamine, as suggested by Funk and Macallum, is being studied. Pitz⁹ claims that lactose used as a supplement to certain foods will prevent the onset of scurvy for 20 weeks. Cohen and Mendel¹⁰ found that with very highly purified lactose scurvy was not retarded. In some preliminary experiments, we have found that our purified lactose was not efficacious in curing scurvy in guinea pigs in the advanced stage. Further study on the specific function of lactose is under way.

⁸ Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

¹⁰ Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

CONCLUSIONS.

From the results of the effect of heat on lactalbumin the data indicate: (1) that high temperatures do not influence the growth-promoting value of lactalbumin when dried *in vacuo* at 55°, or heated in an air oven at 90–100° for 16 hours, or in an autoclave at 15 pounds pressure for 2 and 6 hours, respectively; (2) that the amount of butter fat, whether 5, 18, or 28 per cent when used with a 2 hour autoclaved lactalbumin, has no influence on the rate of growth; (3) that heated lactalbumin is not toxic for young growing rats; (4) that our previous conclusion regarding the excellent growth-promoting value of lactalbumin is further substantiated on the hypothesis that there is a vitamine factor involved which is different from the water-soluble B.

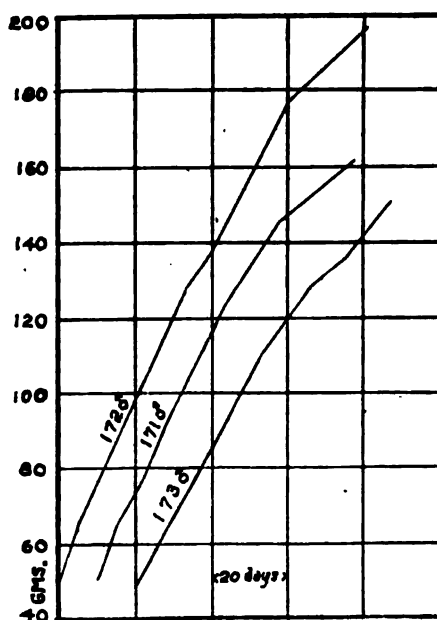


CHART 1. The rats in this group were fed a ration with 10 per cent lard and 18 per cent butter fat, and 10 per cent of lactalbumin protein which had been thoroughly purified by washing with hot water, then digesting a number of times with hot alcohol, and finally washing with ether. This lactalbumin was then dried *in vacuo* at 55–60° for 12 hours. Normal growth resulted.

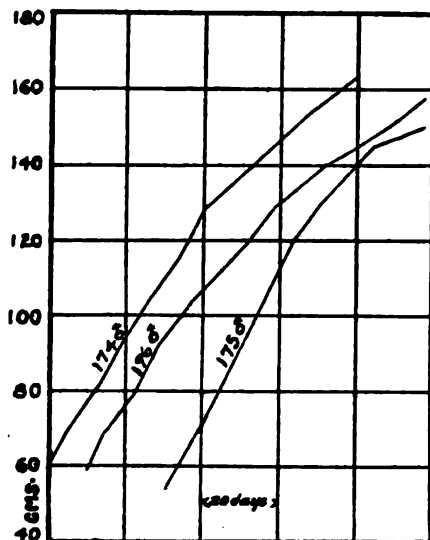


CHART 2. The diet for these rats was the same as that for the animals in Chart 1, except that the lactalbumin was heated at 90-100° for 16 to 18 hours in an air oven. There was very little difference in the rate of growth between this group and that fed the unheated lactalbumin.

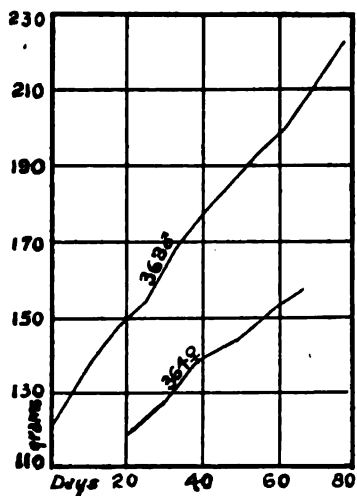


CHART 3. The diet differed from the control ration (Chart 1) in having 10 per cent of protein from lactalbumin which had been heated in the autoclave for 2 hours at 15 pounds pressure.

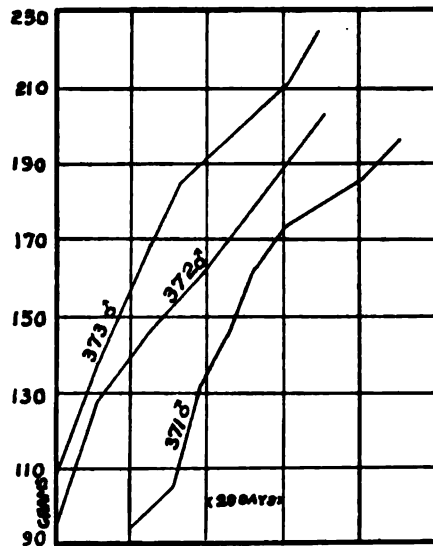


CHART 4. The ration was the same as that fed to the rats for Chart 3, except that only 5 per cent of butter fat and no lard were used.

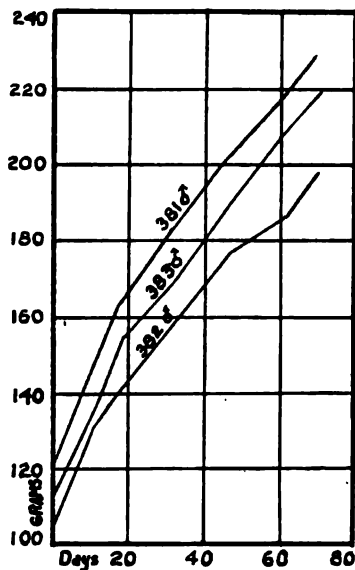


CHART 5. The ration used for these rats was the same as that fed to the group represented in Charts 3 and 4, except for the increase in butter fat to 28 per cent.

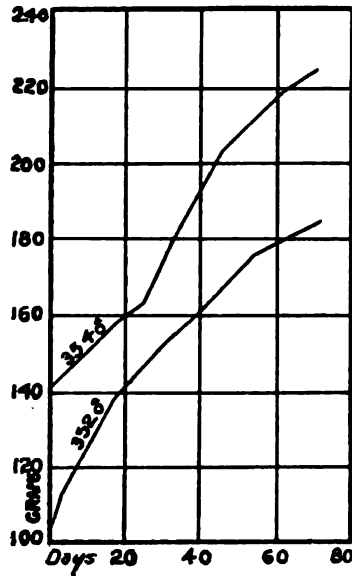


CHART 6. The ration differed from the control diet (Chart 1) and those for Charts 2 and 3, in having 10 per cent protein from lactalbumin which had been heated in the autoclave for 6 hours at 15 pounds pressure.

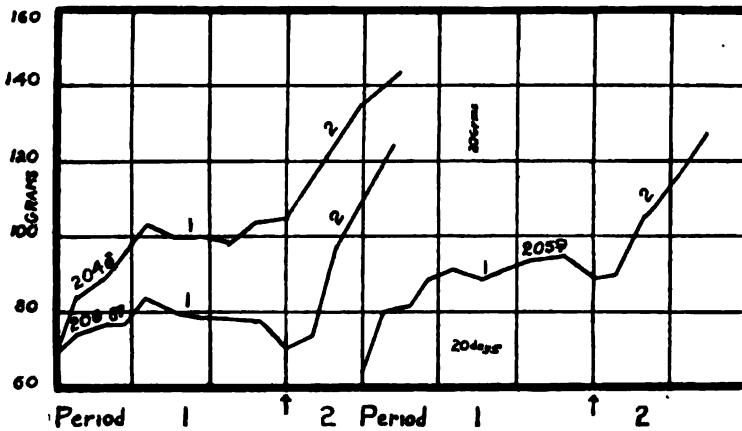


CHART 7. The diet for Period 1 was composed of unheated lactalbumin protein, 10 per cent; butter fat, 18 per cent; lard, 10 per cent; hot alcohol extract of wheat germ, 5 per cent; and starch to make up the balance. In Period 2, part of the starch was replaced by 24.6 per cent of purified lactose; otherwise the diet was the same as in Period 1. This shows that the addition of lactose stimulated growth.

MAINTENANCE AND PRODUCTION VALUE OF SOME PROTEIN MIXTURES.*

BY E. B. HART AND H. STEENBOCK.

WITH THE COOPERATION OF F. LETCHER.

(From the Department of Agricultural Chemistry, University of Wisconsin,
Madison.)

(Received for publication, April 8, 1919.)

It is too well known to require further amplification that the efficiency for growth of a protein mixture will depend upon the qualitative and quantitative make-up of its amino-acid content. The supplementing effect of specific amino-acids for certain definite proteins has been especially studied by Osborne and Mendel,¹ but up to the present time very little data have been accumulated on the efficiency for growth of practical protein mixtures in common use, both in human and in animal nutrition. Some earlier studies in this laboratory by McCollum² disclosed the relatively low efficiency for growth of the cereal grain proteins when fed alone or in mixtures and a much higher utilization of casein and milk proteins.

In addition to the above work data have been collected³ which showed considerable variation in the utilization of protein mixtures from plant sources for milk production which, in its physiological demands, may be correctly compared with growth phenomena. All these studies have a decided practical bearing as they show how a greater utilization of a poor protein mixture can be accomplished by adding to it some single protein or a mixture of proteins with proper supplementing qualities.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325; 1916, xxv, 1; 1915, xx, 351; 1917, xxix, 69.

² McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

³ Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239; 1916, xxvi, 457; 1917, xxxi, 445; 1918, xxxv, 367.

In popular discussions of nutrition problems it is all too common practice at the present time to condemn the cereal grains or other seed products in respect to their protein efficiency. Such condemnation would be warranted if a single cereal grain or a mixture of cereal grains served as the sole diet, but such is rarely the case. What is needed at the present time is definite knowledge as to what products and in what amounts efficient supplementary effects can be secured so that a distinct improvement in the utilization of the proteins of cereals may be brought about. Further, the production value of protein mixtures should be given just as definite an expression as has been given to the net energy values of feeds. True, the production value of proteins, numerically expressed, will be modified by the nature of the protein mixture, by the proportions of proteins from varying sources in the mixture, and by the plane of protein fed. All these factors complicate the problem. For example, the net energy value of 100 lbs. of corn-meal when fed to ruminants is 88.8 therms, which becomes a fixed and definite figure under any conditions of feeding corn-meal to a ruminant. But the production value of the proteins of corn-meal when fed alone to swine is approximately 25; that is from 100 lbs. of corn proteins 25 lbs. can be retained for growth, in addition to maintenance, and this figure 25 would be materially increased if the corn proteins were fed with milk proteins, but with the protein intake at the same level as when corn proteins were fed alone. Further, the figure would be increased if in feeding corn proteins the plane of protein intake were lowered, and *vice versa*. These facts make the problem of determining the production value of proteins a somewhat complex one.

EXPERIMENTAL.

In the use of natural materials such as milk as a supplement to grains there is a marked increase in the nitrogen retention of the mixture by a growing animal. This is interpreted as an improvement in the character of the amino-acid mixture ingested. In the present state of our knowledge an experiment on growth with a grain plus milk is open to the criticism that a grain ration may have been greatly improved by the milk supplement through the addition of extra mineral matter, fat-soluble vitamins, etc..

which are recognized as grain deficiencies. It is yet to be determined whether the inferior showing for growth made by the proteins of cereal grains when fed alone is to be attributed to their deficiency in other factors of nutrition such as the vitamins and mineral matter. To determine the validity of this criticism and to standardize our experimental procedure, pigs of 50 to 75 lbs. in weight have been fed grain mixtures alone and grain mixtures to which were added the most likely deficiencies of the grains other than poor proteins; namely, butter fat as a source of the fat-soluble vitamins, and mineral matter.

In all these test experiments, as well as those which followed, the procedure has been as follows: Vigorous pigs of 50 to 75 lbs. in weight, selected from the Station herd, were given a preliminary starch feeding period of 4 days. At the end of this time they were placed in metabolism cages by which a balance of intake and outgo of nitrogen was determined over a period of 24 days.

There is positive evidence, as shown in experiments on Pigs 30 to 33, Table I, that over a period of 24 days accurate data on the protein efficiency of grains can be secured without vitamins and mineral additions. Butter fat and mineral additions to a corn-meal-oil-meal mixture did not increase the percentage of nitrogen retained as compared with the same grain mixture fed without such additions.

There was not always close agreement among individuals in their rates of growth and consequently in the production value of the protein mixtures tested. However, enough experiments were run with a given mixture to give confidence in the data, and from all the data the three highest results were selected for the final record. In all these experiments the plane of protein intake was kept fairly constant and between 12 to 15 per cent. In one instance it was 11.8 per cent of the ration (experiments on Pigs 13 to 15), and in two other cases slightly above 17 (experiments on Pigs 40 to 45). On these planes of protein intake, 12 to 15 per cent, the amount of nitrogen absorbed and retained by a 50 lb. pig was below the maximum possible. This was done purposely, as in no other way could an accurate efficiency figure be obtained. If more nitrogen is ingested than is necessary to meet the maintenance requirements and the maximum protein constructive powers of the animal the excess of nitrogen will appear in the excreta and necessarily lower the percentage of efficiency.

On a corn and milk ration and a 13 per cent protein level (16.4 gm. of nitrogen daily) a 50 lb. pig will retain approximately 10 gm. of nitrogen per day or 60 per cent of the total nitrogen ingested. Even this figure may not be the maximum retention rate, but such a high value was never reached with any other protein mixture tried except with another grain and milk. For this reason we feel certain that the production values below 60 to 65 per cent which were obtained represent the maximum efficiency for maintenance and growth of the particular protein mixture used.

Special attention should be called to the influence in the rations of a roughage such as alfalfa on the production value of a protein mixture. A roughage, necessarily high in fiber, will produce large fecal residues. With a copious absorption by this fibrous residue of increased intestinal secretions, the amount of nitrogen eliminated by way of the gut and consequently lost to the animal is greatly increased. Compare the fecal nitrogen lost in experiments on Pigs 7 to 9, on a corn-milk-alfalfa ration, and experiments on Pigs 25 to 26, on a corn-milk ration. The fecal nitrogen eliminated in the former series was two and a half times as large as in the corn-milk series. Another factor involved in the above consideration is the lower solubility in the intestinal juices of the nitrogen of alfalfa as compared with the nitrogen of corn-meal or milk. With 25 per cent of the dry matter of the ration coming from alfalfa the production value was lowered 20 per cent. A fact of this kind must be taken into consideration in planning rations of high protein efficiency. Where it is extremely desirable and in fact positively necessary to provide roughage in the ration of most animals for long continued physiological well being, the maximum rates of protein construction must be met by the use of higher planes of protein intake. While corn-meal and milk form an excellent protein mixture of high production value, due in part to low fecal residues, yet such a diet, without roughage, would terminate the life of a growing pig in 5 months.

The production values of the *absorbed* nitrogen of the two rations, corn-milk and corn-milk-alfalfa, are not so different. One might be inclined to use this figure as the numerical expression of the production value of the protein mixture ingested. This, we believe, would be a true expression of the nutritive worth of the

proteins, but such figures would be of less direct or practical value. The proteins are always fed in company with the other constituents of the natural material, and if these modify the amount of total nitrogen left to the organism, as is the case where variable amounts of fiber are ingested, then it would seem to us that this fact must be considered in arriving at the production value of any protein mixture as it occurs in natural feeds; consequently in Table I the production value of the protein mixtures is given in terms of total ingested nitrogen, as well as total absorbed nitrogen.

The net energy requirements of a 50 lb. pig for both maintenance and growth as given by Armsby⁴ is 1.38 therms. No data are available on the net energy content of a number of the feeds used in our work. For example, the production value of alfalfa per 100 lbs. for a ruminant is given as 34 therms, for a horse 48 therms, but no figure is given for swine. In our calculations the figure used for alfalfa was that given for the horse and undoubtedly is somewhat too low. In the absence of available data on the energy value for swine of the other feeds used in our work recourse was made to the figures available for the ruminant or horse. Since the net energy of a feed for a ruminant or horse is somewhat lower than that for swine, due to increased losses incident to intestinal fermentation, the calculated net energy of our rations may have been somewhat lower than what it really was. Probably in every case the energy requirement of the experimental animals was fully met. Further, we were guided somewhat by the appetites of our animals and in consequence of this the ration actually consumed invariably provided more net energy than is called for in Armsby's standard for growing swine. In Table I the column of therms provided represents net energy values.

DISCUSSION.

The data disclose some facts of considerable practical importance. Cereal grain proteins of low production value are not appreciably increased in efficiency by supplementing with a corn kernel protein concentrate, such as gluten feed, or by a legume roughage, such as alfalfa; even the proteins of flaxseed meal (oil-

⁴ Armsby, H. P., *The nutrition of farm animals*, New York, 1917, 713.

meal) when constituting 50 per cent of the ration did not increase the efficiency of those of the corn kernel. But when the flaxseed meal proteins constituted 20 to 25 per cent of the ration and the other 75 to 80 per cent came from the corn kernel there was an increased efficiency. The full data on oil-meal (flaxseed) proteins as supplements will be incorporated in a later paper.

The effective supplementing of the cereal proteins, cereal proteins plus alfalfa proteins, or cereal proteins plus cabbage and potato proteins was accomplished by additions of either milk or whey, or meat or fish proteins. The most effective supplementing was with milk and whey proteins (see charts). With but 16 per cent of the nitrogen coming from the whey proteins the efficiency of a corn-alfalfa-whey protein mixture was made nearly equal to that of a similar mixture in which 27 per cent of the nitrogen came from skim milk proteins in place of the whey proteins. The tankage proteins were not quite so effective in their supplementary relations as were those of milk or whey, although the inferiority was slight. Further, it was found that where 18 per cent of the nitrogen of a corn-alfalfa-tankage ration came from tankage the mixture was practically as efficient as where 35 per cent of the nitrogen came from tankage. It is to be expected that variations in the efficiency of the proteins of such commercial products as tankage, fish meal, meat scraps, etc., will occur, dependent upon what proportion of their total proteins is derived from the less active and incomplete albuminoid group of proteins, such as elastin, collagen, keratin, etc.

Meat crisps or fish meal fed with starch and serving as the sole source of nitrogen (experiments on Pigs 34 to 39) showed efficiencies of but 40 per cent, while milk proteins used under similar conditions showed a value of 60.

The protein mixture from corn and milk, or oats and milk showed production values of 60 to 65 per cent, with approximately 25 per cent of the nitrogen derived from skim milk, while the efficiency of a corn and tankage protein mixture, or a barley-tankage protein mixture was about 40 per cent. In the latter case approximately 30 per cent of the nitrogen was derived from tankage. It appears fairly certain that either milk or meat will supplement the proteins of the various cereal grains to approximately the same degree; that is to say, tankage when used to

supplement barley, oats, or corn will yield a protein mixture of similar production value. Likewise milk will supplement any of the cereal grains to a degree of approximately equal efficiency, but to a degree of greater efficiency than will tankage. This being true, then any differences observed in the rates of growth of animals receiving such mixtures will rest upon other deficiencies or limitations in ration or animal. This principle, once firmly established, leaves the choice of the cereal grain to be used in feeding for growth a matter only of cost.

The experiments on Pigs 46 to 54 touch human as well as animal nutrition. A protein mixture drawn from five common sources, namely rice, wheat, corn, potatoes,⁵ and cabbage,⁶ showed a low value for growth when the protein level was brought to a basis comparable to our other experiments, namely 13.3 per cent, by the use of a protein concentrate such as wheat gluten. The production value of this mixture was 19.4 per cent. When the nitrogen of the wheat gluten was displaced by an equal amount of meat crisps nitrogen the efficiency rose to 32.7, and when it was replaced by an equivalent in milk nitrogen it rose to 47 per cent. The efficiency of the latter mixture was not so high as a corn-grain-milk protein mixture alone, due largely to the greater fecal losses involved in the mixed ration containing cabbage.

⁵ Potatoes were sliced and heated in the autoclave in steam at 15 pounds pressure for 20 minutes.

⁶ The cabbage was dried, but uncooked.

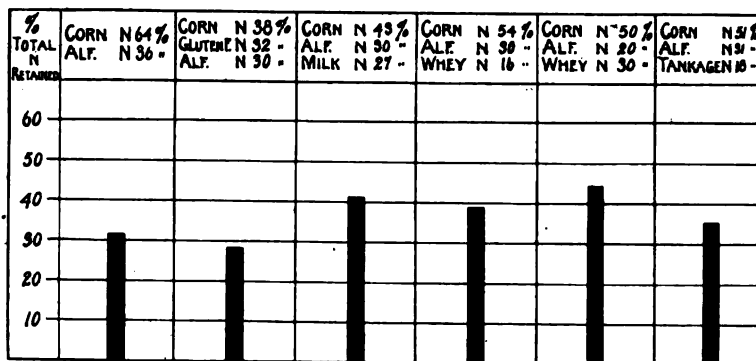


CHART 1. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

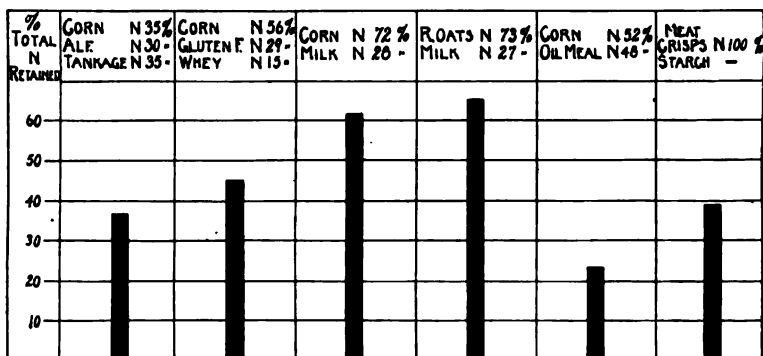


CHART 2. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

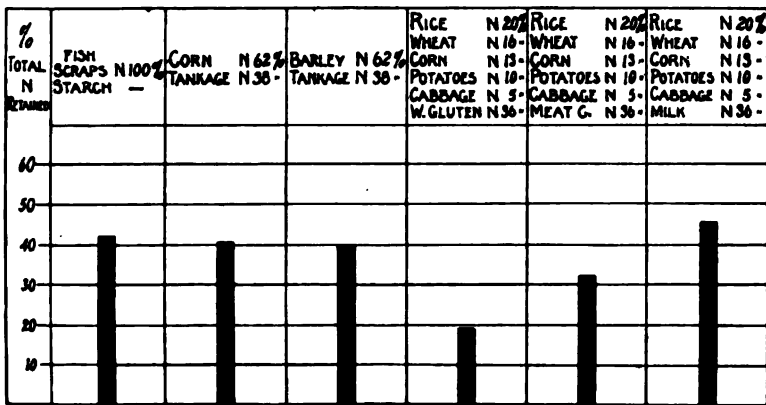


CHART 3. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

TABLE I.
Complete Data on Nitrogen Balance.

[illegible]

7	60	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids. }	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	123.0	101.1	43.7	63.3
8	62	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids. }	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	142.0	97.1	40.0	62.1
9	58	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids. }	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	129.0	106.4	40.9	60.5
Average.....												41.5
10	60	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids. }	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	143.5	84.0	41.8	66.0
11	55	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids. }	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	157.0	86.4	37.7	63.0
12	66	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids. }	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	149.0	94.5	37.7	60.9
Average.....												39.1

TABLE I—Continued.

Pig No.	Live weight.	Feed daily.	Thermas provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.	Absorbed N retained.
	lbs.	gm.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	per cent	per cent
13	53	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.}	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	99.9	112.7	47.4	62.9
14	55	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.}	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	121.4	98.1	45.7	65.3
15	51	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.}	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	124.0	115.1	40.8	78.9
Average.....												44.6
16	62	497 corn. 175 alfalfa. 28 tankage.	1.38	8.45 4.97 2.94	16.36	102.25	14.6	392.6	160.0	99.1	34.0	57.3
17	72	568 corn. 200 alfalfa. 32 tankage.	1.38	9.65 5.68 3.36	18.69	116.81	14.6	448.5	170.0	120.4	35.2	56.7
18	58	568 corn. 200 alfalfa. 32 tankage.	1.38	9.65 5.68 3.36	18.69	116.81	14.6	448.5	172.4	105.2	38.1	61.5
Average.....												35.8

19	53	350 corn. 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	145.6	110.5	36.4	57.1
20	60	350 corn. 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	152.0	97.9	33.0	61.0
21	46	350 corn. 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	160.1	87.1	38.6	64.0
Average.....												37.7
22	70	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	44.6	166.3	46.9	52.9
23	73	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	66.8	174.3	44.3	53.3
24	70	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	59.4	163.0	44.0	51.8
Average.....												45.1

30	49	480 corn. 120 oil-meal.	1.69	6.24 5.88	12.12	75.75	12.6	292.8	94.0	125.9	24.9	36.6
31	48	480 corn. 120 oil-meal.	1.69	6.24 5.88	12.12	75.75	12.6	292.8	79.9	140.8	24.6	33.8
Average.....												
32	40	400 corn. 100 oil-meal. 25 butter fat. 8 Ca acetate. 5 NaCl.	1.69	5.20 4.90	10.10	63.10	12.6	242.4	57.2	125.6	24.5	32.1
33	45	400 corn. 100 oil-meal. 25 butter fat. 8 Ca acetate. 5 NaCl.	1.69	5.20 4.90	10.10	63.10	12.6	242.4	64.8	119.7	23.8	32.6
Average.....												
34	42	135 meat crisps. 565 corn starch.	1.78	15.6		97.5	13.9	374.4	14.5	215.8	38.4	40.0
35	43	140 meat crisps. 560 corn starch.	1.78	16.2		101.2	13.0	388.8	20.0	210.3	40.7	42.9
36	56	140 meat crisps. 560 corn starch.	1.78	16.2		101.2	13.0	388.8	21.1	214.9	39.3	41.5
Average.....												
												39.5

TABLE I—Continued.

Pig No.	Live weight. lbs.	Feed daily. gm.	Therma provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.		Absorb- ed N retained.
				gm.	per cent						per cent		
37	55	200 fish scraps. 500 corn starch.	1.77	16.6	103.7	14.8	398.4	50.2	179.4	42.3	48.4		
38	60	200 fish scraps. 500 corn starch.	1.77	16.6	103.7	14.8	388.4	48.3	180.0	42.7	48.5		
39	42	176 fish scraps. 424 corn starch.	1.53	14.6	91.2	15.2	350.4	76.4	131.9	40.5	51.8		
Average.....													
40	53	535 corn. 65 tankage.	1.55	10.4 } 16.7 6.3 }	104.3	17.3	400.8	121.7	117.8	40.2	57.7		
41	63	535 corn. 65 tankage.	1.55	10.4 } 16.7 6.3 }	104.3	17.3	400.8	128.1	122.4	37.5	55.1		
42	56	535 corn. 65 tankage.	1.55	10.4 } 16.7 6.3 }	104.3	17.3	400.8	100.5	128.1	43.2	57.4		
Average.....													
43	57	535 barley. 65 tankage.	1.50	10.5 } 16.8 6.3 }	105.0	17.5	403.2	133.5	108.4	40.0	59.7		
44	47	535 barley. 65 tankage.	1.50	10.5 } 16.8 6.3 }	105.0	17.5	403.2	139.5	106.9	38.8	59.4		
45	57	535 barley. 65 tankage.	1.50	10.5 } 16.8 6.3 }	105.0	17.5	403.2	135.8	104.2	40.4	61.0		
Average.....													

46	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	71.8	177.2	22.0	28.4
47	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	86.5	176.8	14.4	24.1
48	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	69.1	180.7	21.8	27.8
Average.....												19.4

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A REVISION OF THE COPPER PHOSPHATE METHOD FOR THE TITRATION OF SUGAR.

BY OTTO FOLIN AND EUGENE C. PECK.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

(Received for publication, April 29, 1919.)

While preparing for this season's class work in sugar titrations according to the method of Folin and McEllroy¹ it was found that the salt mixtures prepared from the same lot of chemicals by different individuals did not give identical results. Many of these salt mixtures behaved very much like those recently obtained by Haskins² when he used sodium thiocyanate instead of potassium thiocyanate. When the theoretical quantity of sugar (25 mg. of dextrose) was added the copper was completely reduced in 3 minutes or even less whereas complete reduction should not occur in less than about 4 minutes. The results obtained were the same whether the thiocyanate used in preparing the reagent was the sodium or the potassium salt; but, in the interest of economy, the amount of sodium thiocyanate employed can readily be reduced by 15 to 20 per cent, without encountering red cuprous oxide and without altering the titration value. The variations encountered in the sugar titrations were due partly to the use of granulated disodium phosphate which had lost considerable water of crystallization. The most important cause of the divergent results obtained was due, however, to variations introduced by different individuals in the manner of preparing the salt mixtures. In order to obtain concordant results it is almost indispensable to first mix the phosphate and thiocyanate so as to permit the thiocyanate to abstract enough water from the phosphate to give a solution of the thiocyanate before mixing in the sodium carbonate. The solid salt mixture for the sugar titration of Folin and McEllroy should therefore be made as follows.

¹ Folin, O., and McEllroy, W. S., *J. Biol. Chem.*, 1918, xxxiii, 513.

² Haskins, H. D., *J. Biol. Chem.*, 1919, xxxvii, 303.

Powder in a large mortar 200 gm. of crystallized disodium phosphate ($\text{HNa}_2\text{PO}_4 \cdot 12 \text{H}_2\text{O}$) and sprinkle over it about 50 gm. of sodium thiocyanate (or 60 gm. of potassium thiocyanate). Mix for 10 minutes with mortar and spoon, giving a uniform semi-liquid paste. Add about 120 gm. of monohydrated sodium carbonate (or 100 to 110 gm. of anhydrous carbonate) and mix with mortar and spoon until a rather fluffy granular powder is obtained. Add 5 gm. of the salt mixture to 5 cc. of the copper sulfate solution; if any black specks are formed, even temporarily, the mixing is incomplete; a certain amount of green color is, however, practically unavoidable when this test is applied. If no black coloration is obtained the salt will give substantially correct titration values and can be bottled at once, though we usually leave it in the mortar for a few hours or over night (covered with paper) and then mix once more before transferring to bottles. In stoppered bottles the mixture keeps indefinitely.

The sugar titration according to the directions of Folin and McElroy could however justly be criticised on the ground that the green color obtained when the salt mixture is added to the 6 per cent copper sulfate solution does undoubtedly represent reduction of copper sulfate by the thiocyanate. Correct results are obtained only because the copper sulfate solution has been made strong enough to compensate for the loss due to the thiocyanate reduction. Analogous criticisms can be made of every sugar titration by alkaline copper solutions for in all of these variable amounts of sugar are destroyed by the alkali and the loss of some copper is no more serious than the loss of reducing action on the part of the sugar. In the sugar titration of Folin and McElroy the loss of active copper is visible; it is also both measurable and preventable and the main object of this communication is to describe a modification of the method by which the unimportant but disconcerting loss of copper is eliminated.

The reduction of copper sulfate by the thiocyanate does not occur in alkaline solutions. The thiocyanate is so rapidly soluble in water (or copper sulfate solution) that a certain amount of reduction has taken place before the carbonate-phosphate mixture has had time to make the copper sulfate solution alkaline. The loss of copper thus occurring amounts to about 1.5 per cent. By first adding a little sodium carbonate alone to the copper sul-

fate solution (1 cc. of saturated sodium carbonate) the copper is precipitated and the solution is rendered alkaline and the reaction between the copper sulfate and the thiocyanate is eliminated. The proposed modification requires, of course, that the amount of copper sulfate used for each titration be somewhat diminished; the 6 per cent solution must be reduced to one containing 5.9 per cent of copper sulfate. The reagents required for the revised sugar titration are:

1. The salt mixture prepared as described above, containing 200 gm. of crystallized disodium phosphate, 50 gm. of sodium thiocyanate, and 100 gm. of anhydrous sodium carbonate, or 120 gm. of monohydrated sodium carbonate. For use with the revised titration it is not important to keep the mixture damp and the reagent is more easily weighed or measured if allowed to "weather" until so dry it can be easily poured from one test-tube to another.

2. Approximately saturated sodium carbonate solution (14 to 20 per cent Na_2CO_3).

3. A copper sulfate solution containing 59 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 2 cc. of concentrated sulfuric acid per liter.

Complete reduction of 5 cc. of this copper solution in 4 to 6 minutes is obtained with 25 mg. of dextrose.

Concerning the titration (which is best made in large, hard glass test-tubes) we have little to add to the description given in the original method. It is advantageous to use test-tubes which are dry, or which have been rinsed with alcohol, because with such test-tubes the bumping is much diminished. We now use exclusively special 5 cc. sugar burettes graduated in 0.02 cc. together with accessory tips which are very fine. The burettes are filled by suction.³

Transfer 5 cc. of the 5.9 per cent copper sulfate solution to a test-tube. Add first approximately 1 cc. of saturated sodium carbonate solution. Shake for a moment and then add 4 to 5 gm. of the phosphate-carbonate-thiocyanate mixture. Heat gently with shaking until all the salts have dissolved except for a few isolated particles of sodium carbonate. A clear solution is usually obtained in less than one minute at temperatures which need not exceed 60°C. Add sugar solution or undiluted urine (0.4 cc.

³ These burettes, with accessories, are now made by the Emil Greiner Company, New York.

to 1.0 cc.), heat fairly rapidly to boiling, and then boil *very gently* so as not to drive off too much water. When the mixture begins to bump add a pebble. The following observations, though subjected to some variations, may prove helpful for the avoidance of needless repetitions in connection with the titration.

When 25 mg. of dextrose are added at once, 90 to 93 per cent of the copper will be reduced in 2 minutes and about 98 per cent in 3 minutes. When only 1 per cent of unreduced copper is left, the solution is still so blue that it is impossible to overlook it. This point is most easily demonstrated by adding 1 cc. of carbonate solution and 5 gm. of the solid reagent to 5 cc. of a copper sulfate solution representing a hundredfold dilution of the original copper sulfate solution. With the full required amount of sugar present at the beginning, the boiling solution becomes suddenly turbid within 5 seconds after the boiling point has been reached. If the boiling contents of the test-tube do not thus suddenly become filled with the cuprous thiocyanate precipitate within the first 15 seconds of boiling then less than half of the required sugar has been added and more of the sugar solution or urine should be added without further delay. On the other hand, when an excess of sugar has been inadvertently added at the beginning of the process it is advisable to note the time required for complete decolorization of the copper, for this time (see table) can serve as a guide to the quantity of solution to be introduced at the beginning of the next titration.

TABLE I.

Time of Boiling Required for Complete Reduction of Copper Solution by an Excess of Dextrose.

Dextrose. <i>mg.</i>	Boiling time.	
	<i>min.</i>	<i>sec.</i>
50	0	25
40	0	40
35	0	55
30	1	20 to 30
27.5	1	30 to 55

The only time restriction called for in connection with the final titration is that complete reduction must not occur in less

than 4 minutes boiling; in other words, it makes practically no difference in the result (at the most, 1 per cent) if the boiling period be prolonged to 8 or 9 minutes, provided that the boiling be gentle enough to prevent excessive concentration. The volume of the solution in the test-tube should not become less than 6 to 7 cc.

The modification of the titration of dextrose described above is also applicable to the determination of the lactose in milk, as well as for the titration of other sugars.

THE SCURVY OF GUINEA PIGS.*

III. THE EFFECT OF AGE, HEAT, AND REACTION ON ANTISCOR- BUTIC FOODS.

By ALFRED F. HESS AND LESTER J. UNGER.

(*From the Bureau of Laboratories, Department of Health, New York.*)

(Received for publication, April 17, 1919.)

Recently considerable experimental work has been done on the effect of heat on vitamins, more particularly on the water-soluble vitamin. In a recent article Denton and Kohman (1) come to the conclusion that "ordinary methods of cooking do not perceptibly injure the nutritive value of carrots." These experiments were carried out on rats, which preclude a satisfactory consideration of experimental scurvy.

In the following experiments guinea pigs weighing about 200 to 250 gm. were employed. As will be seen from Chart 1, it was found that in the course of cooking, carrots lose a very large part of their antiscorbutic potency; that whereas 35 gm. of carrots were able to protect a guinea pig from scurvy when added to its ration, the same amount of carrots which had been cooked for about 45 minutes had lost its potency in this respect. An attempt was made to prevent this loss by acidulating the water by the addition of vinegar. As will be noted from Chart 3, however, the animals came down with scurvy within the usual period.

Further experiments demonstrated the fact that from a nutritional standpoint carrots cannot be looked upon as a uniform article of diet; that there is a marked difference in various lots of carrots and probably also of other vegetables, according to whether they are fresh and young, or are old. It was found, for example, that if, instead of employing the carrots which were ordinarily fed

* A preliminary report of this work was published in the *Proc. Soc. Exp. Biol. and Med.*, 1919, xvi, 52.

to our laboratory animals, we gave the same amount of fresh young carrots, plucked only a few days previously and cooked, not only did the animals not develop scurvy, but that they gained steadily in weight for a long period (Chart 2). These carrots could be boiled only for about twenty minutes, as they began to disintegrate if cooked longer; they were then allowed to stand in the boiled water for about one-half hour. Old carrots have therefore a twofold disadvantage in that they contain less antiscorbutic vitamins than the young vegetable, and that they require additional boiling, which still further decreases their limited supply of this essential food factor.

The freshness and age of the vegetables sufficed also to enable them to retain their antiscorbutic potency after dehydration. In previous articles (2, 3) it was stated to have been our experience not only in animal scurvy, but in human scurvy as well, that dehydrated vegetables as purchased in the open market or prepared in the household were without noticeable value in the cure of scurvy. Under favorable conditions certain vegetables can withstand dehydration. This has been conclusively shown by Holst (4) and by Cohen and Mendel (5) in connection with cabbage, and in relation to tomatoes by Givens and McClugage (6). After noting the marked difference between old and young carrots in their ability to withstand the cooking process, we decided to make a similar test in regard to drying, and accordingly had some carrots dehydrated the day they were plucked.¹ It was found that a *per capita* daily allowance of 4.5 gm. of the dried carrots added to the ration was sufficient not only to protect pigs against scurvy but to cure them of this disorder (Chart 3). This amount is equivalent to 35 gm. of fresh carrots, the same quantity which failed as an antiscorbutic when the commercially dehydrated carrots were employed. The dehydrated carrots were kept at room temperature in sealed cartons for a month before the experiment was begun, and had been dried for about 3 months at the end of the test.

This factor of the freshness and age of vegetables or fruit has not been considered in relation to their content of the antiscor-

¹ These vegetables were furnished and prepared by Mr. C. Eckroth. The dehydration was carried out by the Mrs. Oliver Harriman Food Research Laboratories, the same plant which dehydrated the vegetables referred to in the previous papers.

butic or other essential food factors. From the above experiments it is evident that it plays an important rôle, and that in the course of aging at least the antiscorbutic vitamine is greatly lessened. It will have to be given consideration in judging the results in experimental scurvy. This variable factor renders it very difficult to prepare a table of the comparative antiscorbutic value of various foods. Where this is attempted, as recently by Chick and Hume (7), all the vegetables and fruits should be uniform in their development and freshness. It is evident that it becomes a matter of prime importance for the dehydrating industry to make use of such vegetables. It may come to pass that by observing this precaution manufacturers will attain the goal of furnishing a product comparable to the raw food. It should be mentioned that in our experiments there was an additional factor to be considered; namely, that the vegetables were not only fresh but young, and therefore more cellular than the older vegetables.

Experiments were carried out to determine whether the water in which the carrots were cooked contained the antiscorbutic factor. It has been reported by Daniels and McClurg (8) that the liquor from cooked beans contains water-soluble vitamine. Our experiments showed that the water in which the carrots were cooked was of little or no value in protecting guinea pigs from scurvy. The water was given, as will be seen from Charts 4 and 5, in the amount of 40 cc. *per capita* daily, but did not delay the onset of the disease. This addition to the dietary seemed to prevent the marked loss of weight, probably due in part to its salt content. No appreciable difference was noted between the liquor in which old or young carrots had been cooked (Charts 4 and 5).

In the experiments carried out at the Lister Institute by Chick and Hume and other workers, dried milk has been made use of as part of the standard diet in bringing about scurvy in guinea pigs. This food was added to the dietary in order to render it complete and because it was found that this milk was devoid of antiscorbutic power. The inference should not be drawn from these experiments, however, that all dried milk is the same in this regard. It would be an error to infer that milk necessarily loses its antiscorbutic potency when it is reduced to a dry state. In mak-

ing an experimental test of this question we employed a brand of milk which had been dried at a temperature of 116°C. for a period of a few seconds. This milk powder was diluted with eight volumes of water and given to the equivalent of 80 cc. of fresh milk for each guinea pig. This amount was selected as it had been found in previous experiments that 80 cc. of fresh raw milk sufficed to protect a guinea pig from scurvy.

Numerous experiments of this kind were carried out. One of them is shown in Chart 5. It will be seen that five guinea pigs which developed scurvy on a diet of hay and oats and 40 cc. of carrot water were rapidly cured, and gained in weight when the dry milk was added to their dietary. This and similar experiments led to the conclusion that milk which has been dried in this manner (the Just-Hatmaker process) loses little of its antiscorbutic factor. This inference was confirmed by finding that infantile scurvy could be cured by giving dried milk of this variety (2).

In a preliminary communication we reported that canned tomatoes are an excellent antiscorbutic (9). This opinion has been borne out by tests on animals as well as clinical experience. In a recent publication on infantile scurvy it was shown that one ounce of canned tomatoes is sufficient to protect an infant from scurvy and that this food is an excellent antiscorbutic for use in infant feeding and can well replace orange juice, which is so much more expensive. We have carried out an extended series of experiments to ascertain the amount of strained canned tomatoes which it is necessary to add to our ration to protect the guinea pig. It will be seen (Chart 6) that when 1.5 cc. of this food is added to the dietary of each pig scurvy supervenes, but that when this amount is increased to 5 cc. daily, the animals recover and continue to gain in weight for a period of months. Further experiments showed that when only 3 cc. *per capita* are used some of the animals developed scurvy, whereas others escaped this disorder. If the tomato is boiled it loses somewhat in efficacy, although even under these conditions 5 cc. were found to be sufficient to protect the pigs (Chart 7). It may be added that the tomatoes which were made use of had been canned almost one year previously.

In Chart 7 graphs are reproduced which are composites of three series of experiments; one in which 10 cc. of canned tomato were added to the dietary, another in which 30 cc. were added, and a third in which 60 cc. were given. These experiments were prolonged for 4 to 6 months in order to ascertain whether any symptoms of scurvy would eventually become manifest. All animals, however, remained entirely free from this disorder and gained steadily in weight. The group which received only 10 cc. of tomato thrived just as well as that which received 60 cc., showing that the antiscorbutic quota was entirely satisfied by the smaller amount. It may be added, in this connection, that it was found by tests on pigeons that canned tomato contained considerable of the "antineuritic vitamine," and that pigeons suffering from polyneuritis could be cured by giving them 5 cc. daily of this foodstuff.

It has been stated by Harden and Zilva (10) that when orange juice is rendered slightly alkaline it loses its antiscorbutic potency. This proved to be the case under certain conditions. This question was investigated in relation to canned tomato and to orange juice, and it was found that shortly after having been rendered 0.05 N alkaline to phenolphthalein neither of these foods had lost an appreciable amount of its antiscorbutic power. 5 cc. of alkalinized tomato were still able to protect a guinea pig from scurvy (Chart 8), and 5 cc. *per capita* daily of freshly alkalinized orange juice were able to cure a group of pigs which had developed scurvy on another diet (Chart 9). This is in conformity with the efficacious results obtained in human scurvy by the intravenous injection of boiled orange juice which has been rendered faintly alkaline (11). If, however, 24 hours were allowed to elapse between the alkalinization and the feeding, instead of only $\frac{1}{2}$ to 1 hour, then a considerable amount of the antiscorbutic factor was lost. This is well illustrated in Chart 9, where, after the scurvy had been cured by means of freshly alkalinized orange juice, it redeveloped when we gave an equal amount of orange juice which had been alkalinized 24 hours previously. The same rule seems to hold for alkalinization as for heating; *i.e.*, the length of time the antiscorbutic food is subjected to the deleterious influence is fully as important as the intensity of the process.

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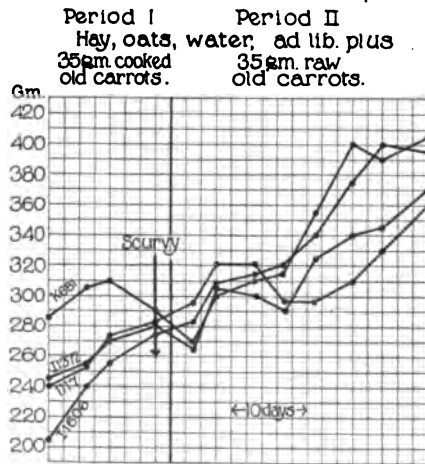


CHART 1. Showing the destruction of the antiscorbutic vitamine by cooking. The raw and the cooked carrots were of the same lot. The vegetables were cooked in an open vessel for about 45 minutes. (Scurvy developed in all 4 animals between the 14th and 16th days.)

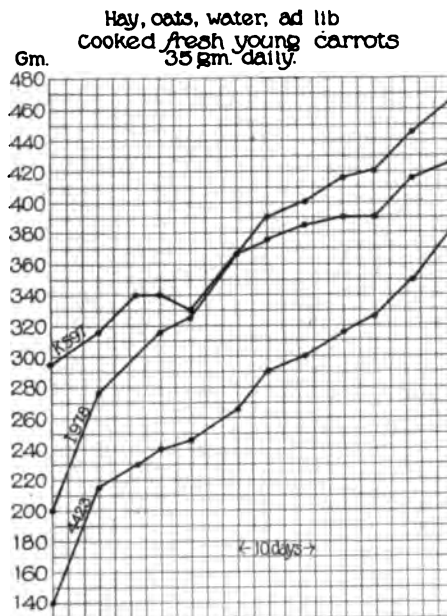


CHART 2. Guinea pigs were given the same amount *per capita* of cooked carrots as in the previous experiment (Chart 1), the only difference being that in the first test the carrots were old, whereas in the second they were young and fresh, having been plucked only a few days before being fed. No scurvy developed.

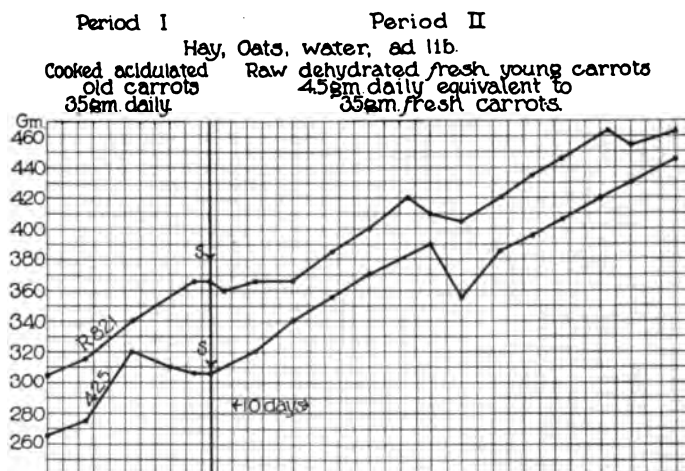
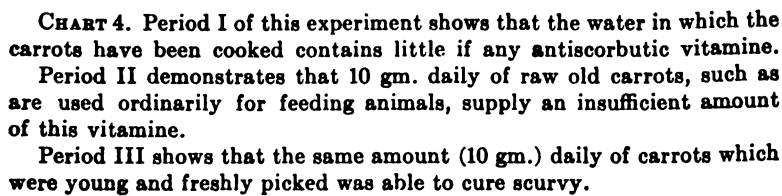


CHART 3. An attempt was made to retain the antiscorbutic vitamine by means of adding 10 per cent of vinegar to the water in which the old carrots were boiled. This failed, however, the animals developing scurvy.

The second or curative part of this experiment shows that if the carrots are young and fresh they will withstand dehydration. In a previous report it has been shown that this amount of commercially dehydrated carrots failed to protect guinea pigs. The carrots were dehydrated the same day they were plucked, at a temperature of about 71°C.



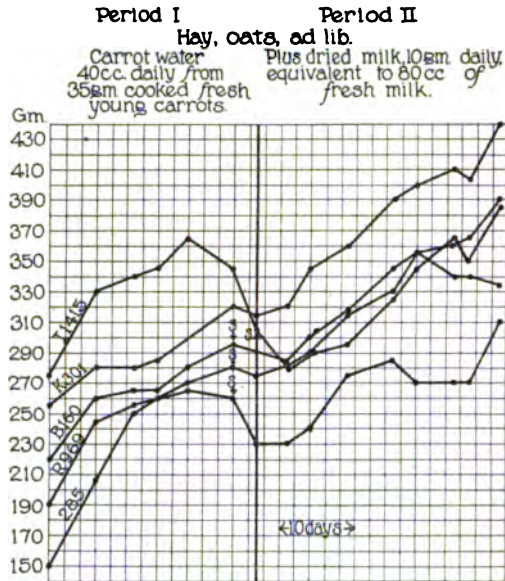


CHART 5. These guinea pigs developed scurvy in spite of receiving a large quantity of the water in which young carrots had been cooked for only 20 minutes. It will be noted, however, that although the pigs developed scurvy they did not lose weight, as is usually the case.

After they had developed definite scurvy they were given in addition the equivalent of 80 cc. of a dried milk prepared by being heated to about 116°C. for a few seconds. The addition of this milk to the diet cured the scurvy, showing that it had largely retained its antiscorbutic vitamin.

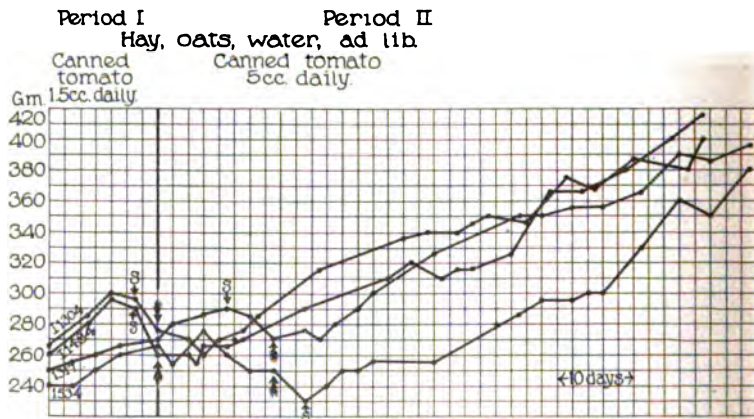


CHART 6. Guinea pigs were fed 1.5 cc. daily of canned tomato, in addition to their diet of hay, oats, and water. This was insufficient to protect them from scurvy. When the amount of tomato was increased to 5 cc. all symptoms of scurvy disappeared and they gained steadily in weight.

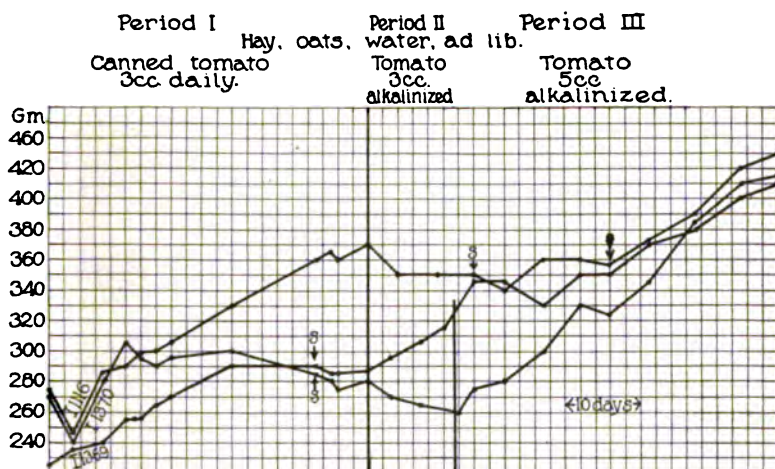


CHART 8. This chart shows that alkalization does not destroy the antiscorbutic vitamine in canned tomato if it is fed soon after the sodium hydroxide is added. In two of the animals, indeed, it seemed to bring about temporary gain in weight. The tomato was rendered 0.05 N alkaline to phenolphthalein.

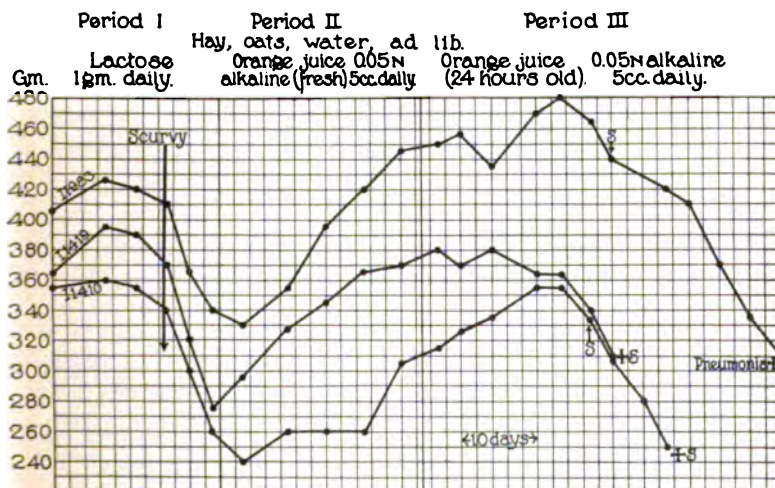


CHART 9. Animals which developed scurvy on a diet of hay, oats, and water plus lactose were cured by means of adding freshly alkalized orange juice.

Period III of this experiment shows that when this alkalized orange juice was allowed to stand in the refrigerator for 24 hours it lost its antiscorbutic potency and was unable to protect the guinea pigs from scurvy.

STUDIES OF EXPERIMENTAL SCURVY.

EFFECT OF HEAT ON THE ANTISCORBUTIC PROPERTIES OF SOME MILK PRODUCTS.*

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The recent work of Chick and Hume,¹ Cohen and Mendel,² Hess and Unger,³ and Givens and Cohen⁴ gives full support to the earlier views of Holst and Frölich⁵ that scurvy is the result of a deficiency of some nutritive factor in the diet. If we understand by the term vitamine the chemically unknown substances of food origin essential for the normal performance of the function of animal life there is perfect propriety in applying to the above mentioned factor the name antiscorbutic vitamine. As polyneuritis is caused by a lack of the water-soluble vitamine (antineuritic vitamine) and xerophthalmia by a lack of the fat-soluble vitamine (antixerophthalmic vitamine), scurvy is caused by a lack of the antiscorbutic vitamine.

Opposed to this view of the etiology of scurvy was the theory of McCollum and Pitz⁶ which postulated that scurvy is related to

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

¹ Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1916-17, x, 141. Chick, H., Hume, M., and Skelton, R. F., *Lancet*, 1918, i, 1.

² Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

³ Hess, A. F., *Am. J. Dis. Child.*, 1917, xiv, 337. Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 479, 487; *Am. J. Dis. Child.*, 1919, xvii, 221.

⁴ Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127. Givens, M. H., and McClugage, H. B., *ibid.*, 1919, xxxvii, 253.

⁵ Holst, A., and Frölich, T., *J. Hyg.*, 1907, vii, 634; *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1; *ibid.*, 1913, lxxv, 334.

⁶ McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxii, 29.

intestinal putrefaction and retention of feces. As pointed out by Chick and Hume, the conclusions of the aforementioned investigators were based on data obtained from experiments where a vitiating factor was constantly operative, in that all the experimental animals were allowed milk *ad libitum*. As milk contains the antiscorbutic vitamine, the incidence of scurvy would consequently be dependent not only on the animal's idiosyncrasy in liking or disliking milk, but also on any change in experimental conditions which tended to modify the fluid intake. Chick and Hume were able to secure some protection against experimental scurvy in guinea pigs when 50 to 100 cc. of milk were given daily per individual, and when 100 to 150 cc. were given scurvy was entirely prevented. In all cases the milk allowance was superimposed on a basal ration of oats and bran.

This work on the rôle of milk we have repeated and confirmed, entirely preventing the development of scurvy with a diet of rolled oats, hay, and 100 cc. of whole milk per individual per day, and delaying the appearance of scurvy in guinea pigs for 18 weeks by a daily allowance per individual of 30 cc. of whole milk and a diet of rolled oats and dried hay. It is altogether probable, therefore, that the development of the theory of the intestinal origin of scurvy became connected with variations in the milk consumed by animals receiving different treatments.

The fact that Hess⁷ was able to cure scurvy in guinea pigs by intravenous injection of orange juice gives incontrovertible evidence of the existence of an antiscorbutic vitamine. So conclusive has become the evidence for the existence in our foodstuffs of this third class of unknowns that we accept this point of view as now fully established.

The point of view that there does exist a relatively unstable antiscorbutic vitamine in our foods offers a satisfactory explanation of the prevalence of scurvy among infants fed milk of which the origin and heat treatment may have been variable. It opens for study the question of the variation in antiscorbutic vitamine content of milks produced under various conditions. The fact, first demonstrated by Holst and Frölich and further developed by Hess and by Givens and his associates, that mere

⁷ Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 141.

drying of plant tissue destroys considerable quantities of this vitamin, possibly places the feeding of cows for special purposes, as for the production of a milk rich in the antiscorbutic factor, in a new light and calls for investigation. Further, with the recognition of a third class of vitamins, in addition to the fat-soluble (antixerophthalmic) and water-soluble (antineuritic) types, as essential to the life of certain species as man, monkey, and guinea pigs, comes the question of its relation to the life of other species of animals. Does the guinea pig need an abundance of this vitamin and therefore fail on a diet of corn-meal, peas, and dried alfalfa flour,—low in the antiscorbutic factor—while the rat, cow, or pig needs less or none of it, and therefore succeeds on such a ration? These are questions of great interest and fundamental to a complete understanding of all the factors involved in animal nutrition. A recent paper by Harden and Zilva² involves an investigation of this question with special reference to the rat.

The data presented in this paper involve a repetition of some of the earlier experiments conducted in this laboratory and, in addition, demonstrate that commercial condensed milks, commercial milk powders, and milks sterilized for 10 minutes at 120°C. have suffered the destruction of their antiscorbutic vitamin as determined experimentally with the guinea pig.

An excellent description of the symptomatology of guinea pig scurvy is given by Cohen and Mendel.³ Our experience in following the course of this disease coincides very well with their observations. There occurs tenderness of the joints which provokes crying when touched; a progressive swelling of the affected joints, which, if not cured, become permanently hardened into exostoses; and a slowly increased inability to use the hind legs, which finally develops into an apparent paralysis of those parts. At this latter stage the animal lies on its side or back a considerable part of the time, taking the "face ache" position; the lower molars become loose, being easily picked out with a forceps, and in some cases there is an appearance of hemorrhage at the base of the lower incisors. At autopsy the junctions of the ribs and cartilages are enlarged, the bones are easily breakable, and conditions of hemorrhage or congestion are usually noticeable.

² Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 408.

The diet chosen as one invariably producing scurvy in the guinea pig was rolled oats and dried hay,—a mixture of June grass and timothy hay (see Chart 1). Fresh milk in the diet was avoided unless its consumption was quantitatively controlled because of its power, in an oat-hay diet, to prevent scurvy if consumed in large enough quantities. In the absence of hay and with a diet of rolled oats and fresh whole milk, involving an average daily consumption of 47 cc. of milk per individual, the guinea pig will develop scurvy (see Chart 2). In the presence of the hay a somewhat less amount of milk (30 cc.), consumed daily per individual, will prevent or at least greatly delay the onset of scurvy (see Chart 3). These results can be interpreted in two ways: either the hay still contains some of the antiscorbutic vitamine, which in addition to that in the milk and grain is sufficient to prevent the development of the disease, or the hay, as a source of roughage, or of mineral matter as pointed out by Pitz,⁹ improves the conditions for normal nutrition to a degree sufficient for a smaller amount of the antiscorbutic factor to suffice. With a daily allowance of 30 cc. of fresh whole milk per individual, together with a ration of rolled oats and hay,—the latter always fed separately and *ad libitum*—there was a delayed development of scurvy, but absolute protection was not secured (see Chart 3). One animal, after giving birth to young, gradually developed the disease, while the other three animals showed symptoms of enlarged joints only after being on the ration 20 weeks.

Where 100 cc. of whole raw milk were allowed daily per individual and an average daily consumption per individual of 84 cc. was actually recorded with a basal ration of rolled oats and hay, absolute protection from scurvy was secured (see Chart 4). Even at the present writing and after a period of observation of 26 weeks, the animals appear in perfect condition. This work confirms the work of Chick and her associates on the quantitative relation of raw milk consumption to the development of a scorbutic condition in the guinea pig.

With either rolled oats and hay or rolled oats and milk we have been unable to prevent the development of scurvy by the administration of mineral oil. Lot 5, Chart 5, receiving a diet of

⁹ Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

rolled oats and hay was given 1 cc. of mineral oil per individual every other day after the first symptoms of scurvy appeared. They all died of the disease. Lot 6, Chart 6, on a diet of rolled oats and milk *ad libitum* (but an average daily consumption of 47 cc. per individual per day) developed scurvy from which they died at the end of 6 to 8 weeks. They had received 1 cc. of mineral oil per individual on alternate days starting from the time of the inauguration of the experiment. Even with a diet of rolled oats and hay, but with 1 cc. of mineral oil per individual administered daily from the beginning of the experiment, the development of scurvy could not be prevented (see Chart 7). There is but one conclusion possible from these data, namely, that mineral oil, acting as a laxative, cannot *per se* prevent the development of scurvy.

Phenolphthalein administration was also ineffective in preventing the development of scurvy on a diet of rolled oats and hay (see Chart 8). To this group 2 mg. of phenolphthalein per individual were administered on alternate days by gelatin capsule. Whether the administration of the phenolphthalein was begun at the initiation of the experiment (see Chart 9) or after the onset of scurvy symptoms, the final results were the same. All the animals died of scurvy. The more mature animals were as sensitive to the development of scurvy as those less mature and the disease terminated their lives in 5 weeks.

Pitz⁹ had observed that lactose could prevent the development of scurvy in guinea pigs receiving a diet of rolled oats, a salt mixture, and fresh milk *ad libitum*. The fact that again milk was accessible to the animals for *ad libitum* consumption makes the conclusion questionable. The lactose may have increased the milk consumption or possibly may have carried some of the antiscorbutic vitamine, as the product was not especially purified in respect to its vitamine content. The results secured with Lot 10, Chart 10, where unheated lactose (Merck) was used with a diet of rolled oats and hay and with Lot 11, Chart 11, where the same quality and quantity of lactose were used, but after having been heated to 120°C. for 4 hours, are conclusive evidence that lactose in itself is not an antiscorbutic. All the animals in both groups died of scurvy in 4 to 5 weeks. They ate the rations well for 2 weeks as indicated by their increments in live weight.

Of special interest to us was the effect on the guinea pig of certain rations known by long experience to be satisfactory for cattle and swine. Rations consisting of intimate mixtures of rolled oats 84 parts, dried corn-stover 15 parts, and common salt 1 part (see Chart 12); or dried alfalfa hay 25 parts and rolled oats 75 parts (see Chart 13); or dried corn-stover 49 parts, rolled oats 50 parts, and common salt 1 part (see Chart 14); or corn-meal 35.7 parts, gluten feed 14.3 parts, dried corn-stover 49 parts, and common salt 1 part (see Chart 15); all produced scurvy in the guinea pig in from 4 to 5 weeks. Such rations gave no apparent nutritional disturbances over long continued use with cattle or swine. For example, we have reared cattle to maturity and obtained very successful reproduction on a ration of corn-meal 35.7 parts, gluten feed 14.3 parts, dried corn-stover 49 parts, and common salt 1 part. It is true, as far as we are aware, that scurvy has not been reported in cattle or swine, but it is difficult to conceive why any mammal should be exempt from the needs of this class of vitamins, while the guinea pig, monkey, and man are extremely sensitive to its absence or a low supply. Probably it is wholly a matter of different quantitative demands and therefore the rations mentioned may have supplied a sufficient quantity of this nutritive factor for the normal performance of such species as cattle, swine, or rats.

Antiscorbutic Properties of Sterilized Milk.

Frölich¹⁰ and later Chick, Hume, and Skelton¹¹ have called attention to the poverty of heated milks in the antiscorbutic vitamin. Hess and Fish¹² and later Hess¹³ reported a mild outbreak of infantile scurvy caused by the use, for several months, of a diet of cow's milk previously pasteurized at 63°C. for 30 minutes. The scorbutic symptoms disappeared on the restoration of the previously used antiscorbutic, orange juice, or on the substitution of raw milk for the heated milk.

¹⁰ Frölich, T., *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 155.

¹¹ Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

¹² Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385.

¹³ Hess, A. F., *Am. J. Dis. Child.*, 1916, xii, 152.

We have not as yet done any work with pasteurized milk, but have investigated, with respect to their antiscorbutic properties, milk sterilized at 120°C. for 10 minutes, two brands of unsweetened condensed milk, and one commercial brand of skimmed milk powder.

Milk sterilized at 120° for 10 minutes has lost its scorbutic protective powers for the guinea pig receiving a diet of rolled oats and dried hay (see Chart 16). A daily allowance per individual of 100 cc. of milk was made, but not all of this was consumed. The average daily consumption per individual, had it been raw milk, would have adequately protected them against scurvy. As it was, two of the animals died of scurvy at the end of 7 and 9 weeks respectively, after having consumed daily 47 cc. of milk per individual. The other two animals showed severe symptoms of the disease at the end of 4 weeks and had begun to lose weight. Their average daily milk consumption per individual had been 43 cc. At this point 1 cc. of orange juice per individual was administered daily by pipette with rapid recovery and gain in weight, demonstrating the adequacy of the diet in respect to all other factors but the antiscorbutic vitamine. The average daily milk consumption of these animals was much less during the orange juice-free period, but, including the orange juice-free period, the average milk consumption was 79 cc. per day per individual for the entire period of observation.

Antiscorbutic Properties of Unsweetened Condensed Milk.

The brands¹⁴ of unsweetened condensed milk with which we have worked have also lost their antiscorbutic properties. The heat treatment given milks in the milk condensing industry varies considerably during different seasons of the year and also in different localities. A large condensing concern in reference to this matter of heat treatment states as follows:

"The general statement can be made that the pre-heating temperatures vary from 82.5°C. to 98.5°C. and the time from one minute to twenty minutes. The condensing temperature runs from 55°C. to 70°C., the time

¹⁴ Golden Key, Valecia Evaporated Milk Co., Madison, Wis. Carnation, Carnation Milk Products Co., Seattle, Wash.

depending on the amount of milk condensed. The sterilizing temperatures run from 107°C. to 115.5°C., the time varying from twenty minutes to fifty minutes."

This is a more severe treatment than the sterilization of milk at 120°C. for 10 minutes. To test its antiscorbutic properties the condensed milk was diluted with an equal volume of water, making its content in solids equivalent to normal milk. A daily allowance to each animal of 100 cc. of this diluted milk was made, together with the usual ration of rolled oats and hay. The average daily milk consumption, had it been raw milk, would have amply protected against such an early attack of scurvy (see Chart 17). Two of the animals died from scurvy at the end of 3 and 5 weeks respectively. Their average daily consumption of milk per individual was 42 cc. The other two animals developed severe symptoms of the disease at the end of 5 weeks, at which time 1 cc. of orange juice per individual was administered by pipette daily. Rapid recovery followed, with pronounced increase in weight. The average daily consumption of milk per individual was 50 cc. for the entire period. While 30 cc. of whole raw milk on a rolled oats-hay diet offered protection against scurvy for 15 to 18 weeks, yet with these products, over 40 cc. per individual per day offered no protection whatever.

Antiscorbutic Properties of Milk Powder.

Judging from our limited data on milk powders accumulated up to the present time these products have likewise lost their antiscorbutic properties. Probably most of the milk powders on the market today are made by the spray process and here again there is no rigid uniformity among manufacturers as to time and heat treatments in the various stages for the process. One manufacturer informs us that in his plant the skimmed milk is heated for 20 minutes at 94°C. and then condensed in a vacuum from about five to one volume. The milk is next cooled rapidly and held in storage for the powder mill. The condensed milk is then run through a Jensen pasteurizer and heated to 60°C. just ahead of the hydraulic pump which forces the milk through the spray. The powder room is held at a temperature ranging from 70-77°C. The powder lies in this room during the day's run and is taken

out in the evening. Another manufacturer first condenses the milk and then pre-heats it to 49°C. and with the aid of a pump developing a pressure of 2,000 pounds per square inch, forces the spray into the powder room. A continuous current of hot air at an approximate temperature of 149°C. is forced into this room at a rate of 14,000 cubic feet per minute, which, when it comes in contact with the milk spray, immediately loses its high temperature of 140°C. and falls to 82°C. Gradually the temperature of 82°C. is reduced through the various channels of operation to a temperature of 55-65°C.

It is clear that the milk powders on the market have been exposed to heat treatments of varying degrees and probably in most cases of sufficient severity to destroy the antiscorbutic vitamine or at least greatly reduce its quantity. More data, however, are required to establish this point fully. In the light of our experience the report of the United States Public Health Service¹⁵ on British experience in the use of milk powder in infant feeding is confusing. This report indicates a general success with milk powders in infant feeding, although it advises that "The occasional use of fruit juice is desirable."

In the records shown in Charts 18, 19, and 20 but a single brand¹⁶ of milk powder was used. In our first experiments, Chart 18, 10 parts of the milk powder were mixed with 90 parts of rolled oats and the mixture was fed with the dried hay; the latter, as in all other experiments, was allowed in a separate container. Normal guinea pigs of 200 to 250 gm. in weight will consume daily 15 to 18 gm. of the oat-milk powder mixture. This amount of food, containing 10 per cent of milk powder, would mean the consumption of an equivalent in raw milk of but 15 to 18 cc. per day. All the animals developed scurvy and died in 4 to 5 weeks. The allowance of milk powder was insufficient to test adequately the problem in hand.

In our next experiment with the same powder (Chart 19) the milk powder constituted 25 per cent of the oat-milk powder mixture. With a similar daily consumption per individual of 15 to 18 gm. of the oat-milk powder mixture an equivalent of 40 to 45

¹⁵ *Bull. Hyg. Lab., U. S. P. H., No. 473, 1918.*

¹⁶ This brand was a Merrill Soule powder, concerning which no record of heat treatment could be secured.

cc. of raw milk would have been consumed. This quantity of raw milk would have been adequate for at least a long delay in the development of scurvy symptoms. Yet all these animals died with typical scurvy in 5 to 6 weeks.

In Lot 20, Chart 20, the proportion of skimmed milk powder was further increased and made 50 per cent of the oat-milk powder ration. With guinea pigs of 250 to 275 gm. in weight and a daily consumption of 15 to 18 gm. of the mixture per individual, there would be a daily consumption of milk powder equivalent to 75 to 90 cc. of raw skimmed milk. Yet on this liberal allowance, which as raw milk would have permanently protected against scurvy, all the animals succumbed to the disease in 5 to 15 weeks.

The lesson is obvious. Either the results with guinea pigs on experimental scurvy should not be translated to infantile scurvy, or we should follow the wiser course of using some antiscorbutic in conjunction with the exclusive use in infant feeding of such heated milk products as described in this paper.

SUMMARY.

1. On a diet of rolled oats and hay the prevention of scurvy by the use of raw milk will depend upon the amount of raw milk allowed. This confirms the results of Chick and her associates.

2. Such laxatives as mineral oil or phenolphthalein or the sugar, lactose, are not in themselves preventives of scurvy.

3. Milk sterilized at 120°C. for 10 minutes, commercial unsweetened condensed milk, and the commercial milk powder examined had lost their antiscorbutic properties when used in quantities equivalent to an amount of raw milk which would prevent scurvy in guinea pigs on a diet of rolled oats and dried hay.

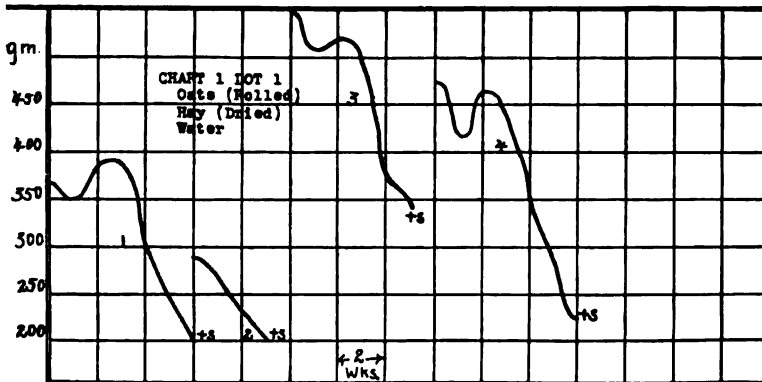


CHART 1. Lot 1 received a diet of rolled oats and dried hay (a mixture of timothy and June grass). These materials were fed separately, the hay being put in the cage uncut. The hay was eaten with a relish. This ration provided all the factors of nutrition, except a good protein mixture and the antiscorbutic factor. Guinea pigs invariably died of scurvy on such a ration.

S = Scurvy.

+ = Died.

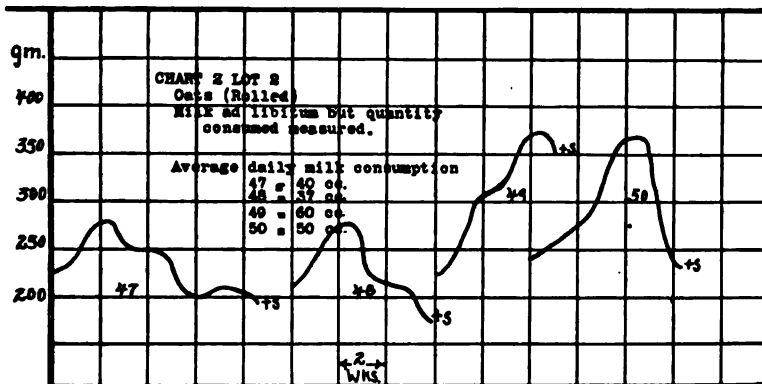


CHART 2. All the animals in Lot 2 died of scurvy. Without the hay and with nothing but rolled oats and milk *ad libitum* (actual consumption of milk shown in chart) they were unable to survive. The quantity of whole milk consumed was undoubtedly too small to act as an efficient scorbutic protector.

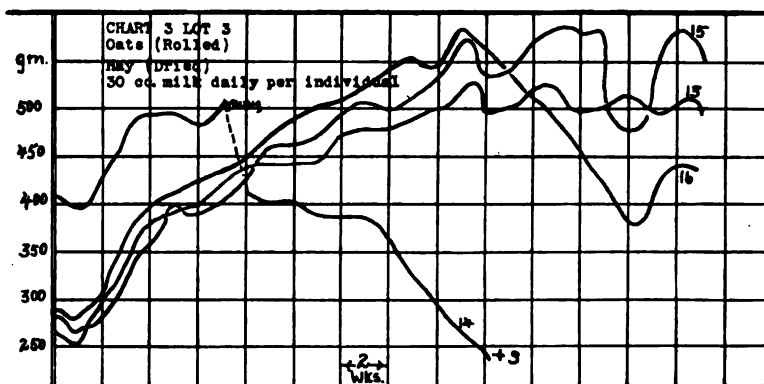


CHART 3. This lot received rolled oats, dried hay, and a small allowance per individual of fresh whole milk. By a daily consumption of 30 cc. of fresh whole milk per individual, there was great delay in the onset of scurvy. The hay probably contributed some of the necessary anti-scorbutic, in addition to roughage, salts, and fat-soluble vitamins, and through this improvement in the ration over a rolled oats-milk diet made possible an effective protection against scurvy with a small supply of milk. Animals 13, 15, and 16 showed enlargement of the joints at the end of 20 weeks, indicating a mild condition of scurvy.

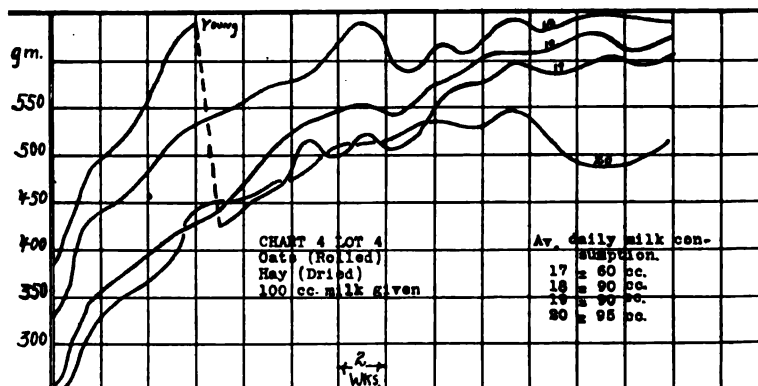


CHART 4. Complete protection against scurvy on a rolled oats-dried hay diet when the fresh whole milk consumed daily per individual equaled 60 cc. or more.

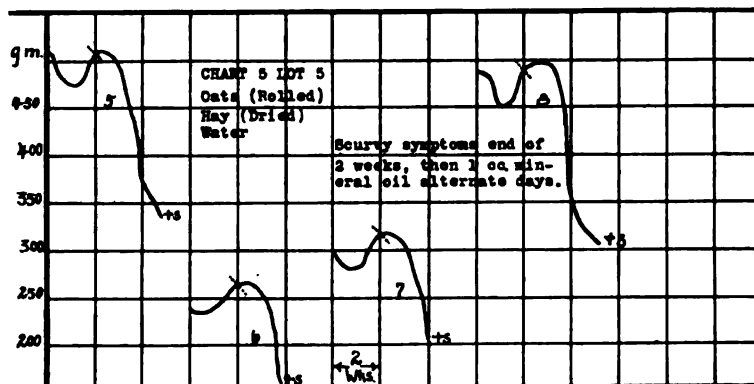


CHART 5. On a diet of rolled oats and dried hay mineral oil did not protect the guinea pig against the course of scurvy after it had developed. The theory that scurvy is primarily related to intestinal disorders is untenable.

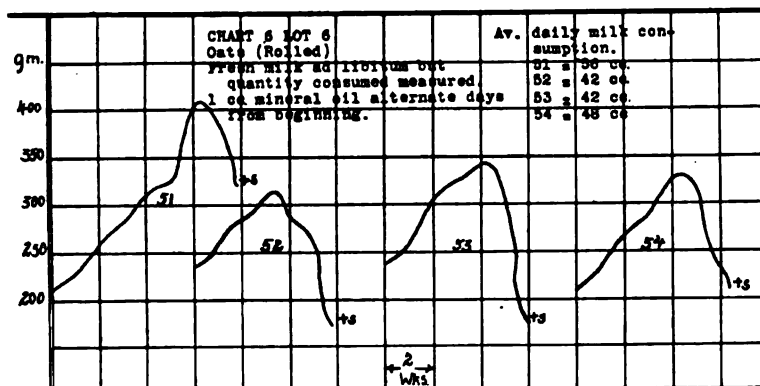


CHART 6. The behavior of this lot is further evidence of the non-protection against scurvy by the use of a laxative such as mineral oil. A diet of rolled oats and whole milk (with an average daily consumption per individual of 42 to 56 cc.) produced scurvy even with an administration on alternate days of 1 cc. of mineral oil per individual. The milk addition improved the ration for growth. Where cures or preventions of scurvy by the use of a laxative have been accomplished on a diet of rolled oats and milk *ad libitum*, such as reported by McCollum and Pitz,⁶ it is probable that a larger consumption of milk occurred than was here observed.

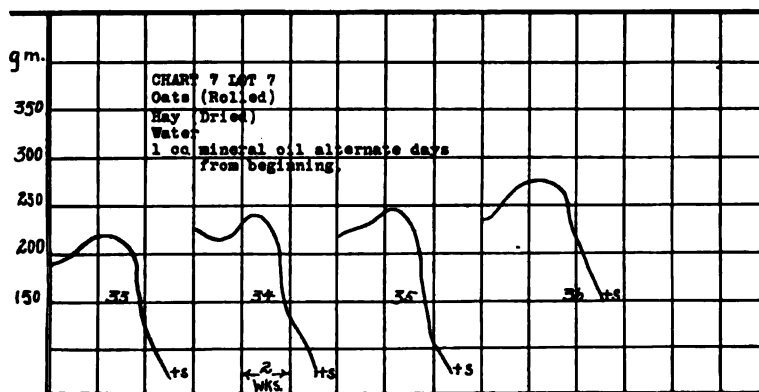


CHART 7. Administration of the mineral oil from the time the animals were placed on the diet of oats and hay was equally ineffective in protection against scurvy (cf. Chart 5).

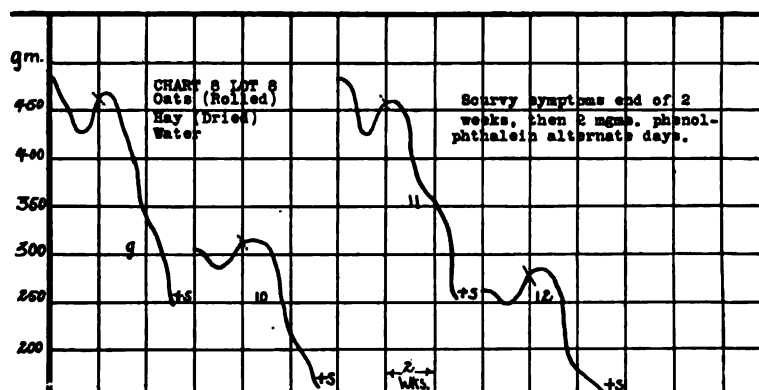


CHART 8. Phenolphthalein was ineffective as an antiscorbutic with guinea pigs receiving a diet of oats and dried hay. Administration of the phenolphthalein was begun after the early symptoms of scurvy appeared.

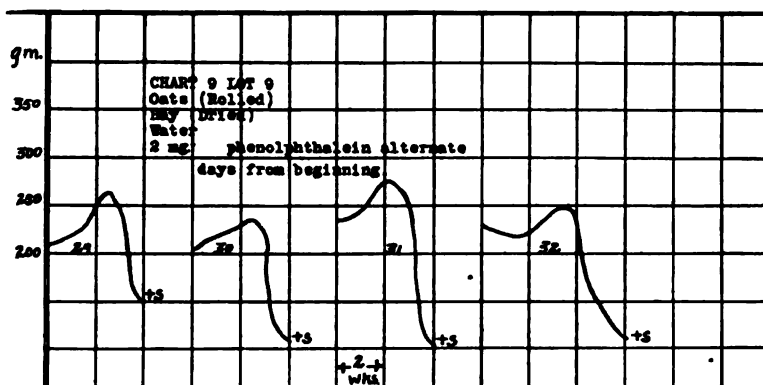


CHART 9. Administration of phenolphthalein on alternate days from the beginning of the experiment failed to protect guinea pigs, receiving a diet of rolled oats and dried hay, against the development of scurvy.

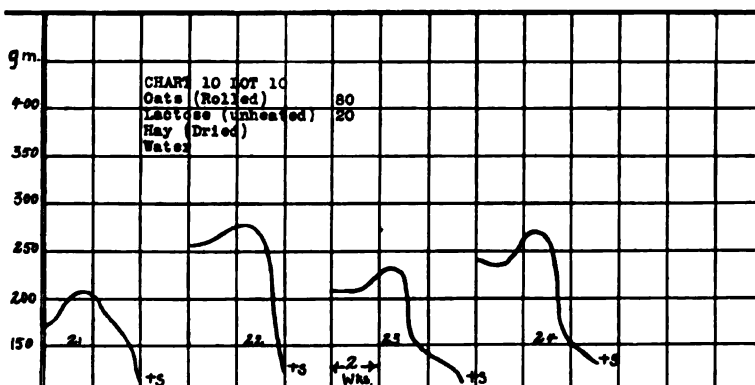


CHART 10. This Chart illustrates the record of guinea pigs receiving a diet of rolled oats, dried hay, and unheated lactose. They all died with scurvy. On a diet of rolled oats, salt mixture, and milk, Pitz⁹ had succeeded in dispelling the symptoms of scurvy by the use of lactose. The probable explanation of that result lies in a larger milk consumption by the animals after lactose administration. This would then mean that recovery was really due to an increased intake of the antiscorbutic vitamine.

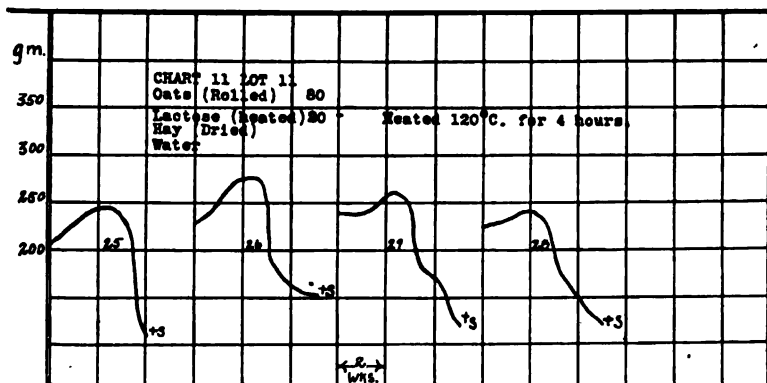


CHART 11. Lactose heated for 4 hours at 120°C., to destroy any anti-scorbutic factor that it might contain, differed in no way from unheated lactose in its effects on the development of scurvy on a diet of rolled oats and dried hay.

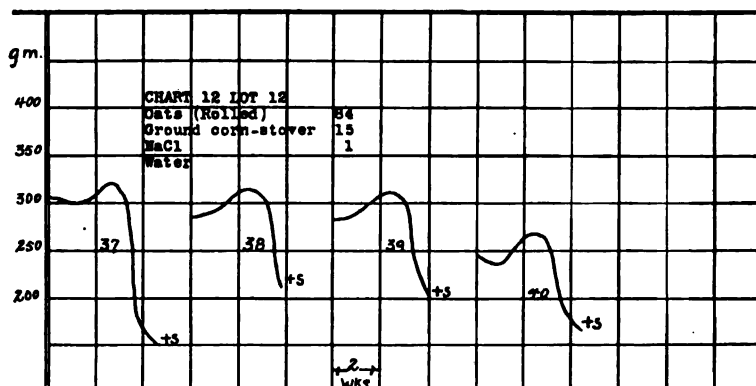


CHART 12. This Chart illustrates how a diet, satisfactory for one species, will fail with another. Cattle or swine could be reared at a slow rate of growth and maintained on a ration of rolled oats 84 parts, corn-stover 15 parts, and common salt 1 part, but this dried material is too poor in the antiscorbutic to prevent scurvy in the guinea pig. This may mean only that cattle and swine demand less of the antiscorbutic factor than do guinea pigs.

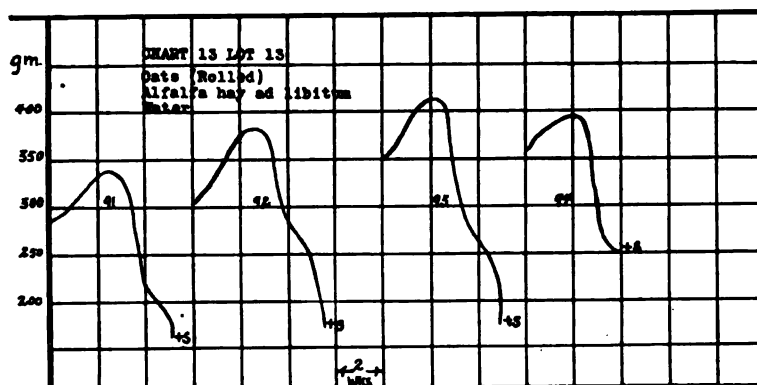


CHART 13. Another illustration of the failure of a dried roughage, such as alfalfa, to protect against scurvy in the guinea pig. A ration of rolled oats and dried alfalfa would be adequate for swine, cattle, or rats, as far as maintenance is concerned, although it would not allow normal growth in swine or rats; but with the guinea pig it leads to scurvy. This is interpreted as a difference in the antiscorbutic demands of the species and as an indication that the rolled oats and dried alfalfa have lost much of their antiscorbutic vitamine in drying or aging.

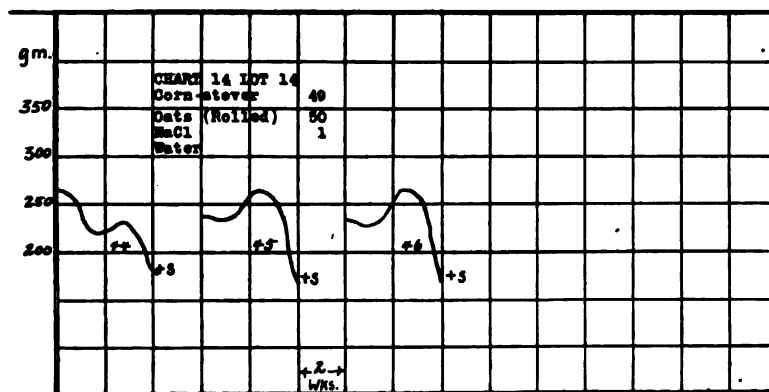


CHART 14. Even a larger proportion of dried corn-stover in the diet (49 per cent as contrasted with 15 per cent, see Chart 12) will not protect the guinea pig against scurvy. The roughages, such as alfalfa or corn-stover, that were used in these experiments were the ordinary dried material stored in the University barn from the crop of 1918.

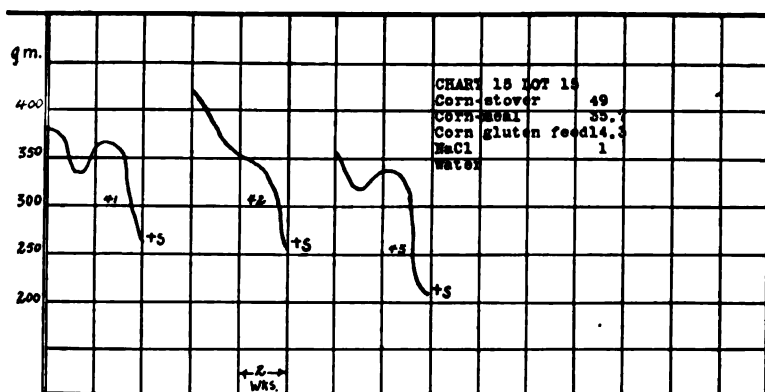


CHART 15. A ration which has grown and maintained cattle for 4 years as the sole diet, but which produced scurvy in the guinea pig. Either cattle are not susceptible to the disease or their demands for the anti-scorbutic vitamine are relatively less.

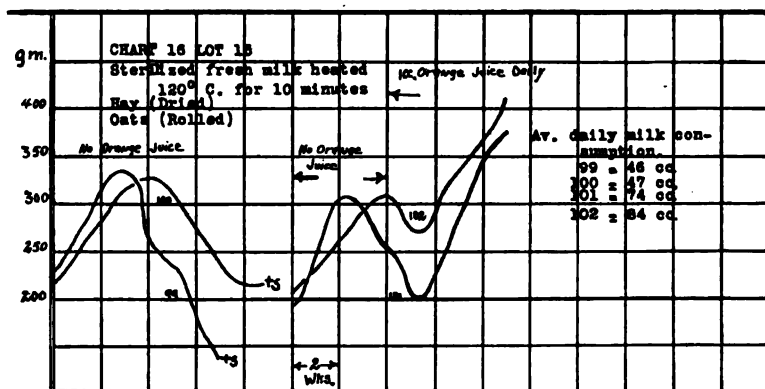


CHART 16. Milk sterilized at 120°C. for 10 minutes has lost or suffered a great reduction in its antiscorbutic content. On a diet of rolled oats, hay (dried), and 30 cc. of fresh milk daily per individual the development of scurvy can be greatly delayed (see Chart 3). Yet, with a similar diet and a consumption of 46 and 47 cc. of sterilized milk scurvy terminated the life of Animals 99 and 100 in 7 to 9 weeks. The curves of Animals 101 and 102 illustrate the recovery from scurvy brought about by a diet of rolled oats, hay, and sterilized milk, but with the daily administration of 1 cc. of orange juice per individual. After the administration of the orange juice and recovery the average milk consumption per day was considerably increased.

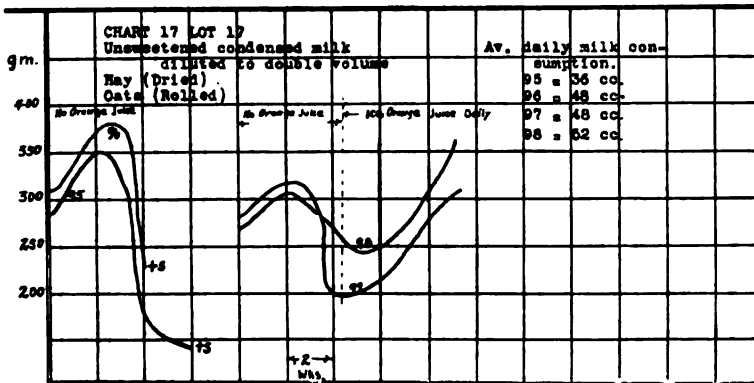


CHART 17. The brands of unsweetened condensed milk used in these experiments had also lost their antiscorbutic properties. The consumption of this milk, diluted so as to be equivalent in concentration to normal fresh milk, was 36 to 52 cc. per individual per day. All developed scurvy in 3 weeks and the disease was allowed to run its course in the cases of Animals 95 and 96. To the other two animals 1 cc. of orange juice was given daily by pipette with complete recovery, except for the permanent enlargement of the joints.

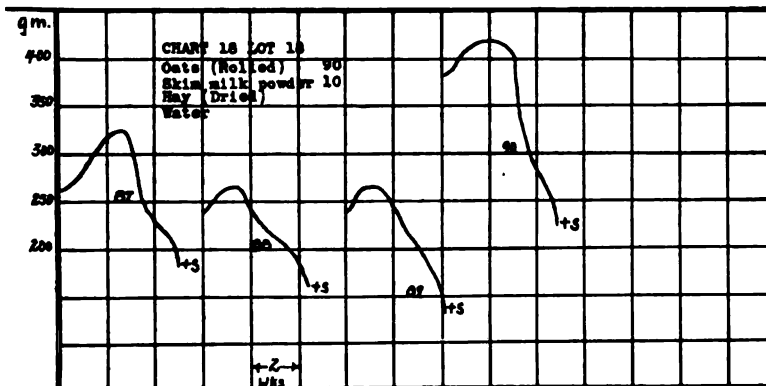


CHART 18. The brand of commercial milk powder examined possessed no protective qualities against scurvy. On a diet of rolled oats, dried hay, and a daily allowance of milk powder, equivalent to 15 to 18 cc. of skim milk per individual, scurvy terminated the life of the animals in 5 weeks.

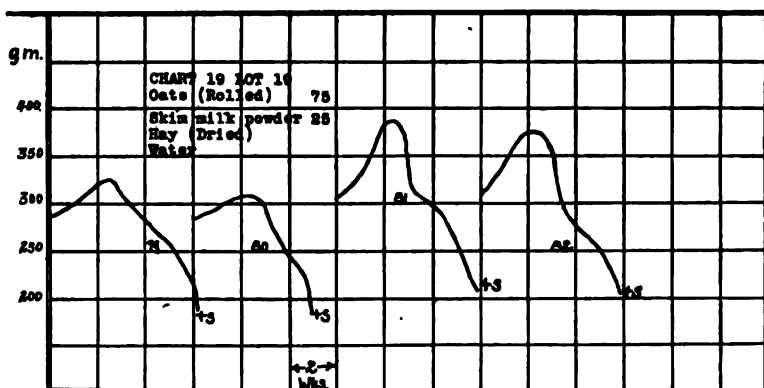


CHART 19. Another failure on milk powder. When the allowance of milk powder was increased to an amount equivalent to 40 to 45 cc. of raw milk per individual per day, with a diet of rolled oats and hay, scurvy rapidly developed and all of this group died of the disease in 5 to 6 weeks.

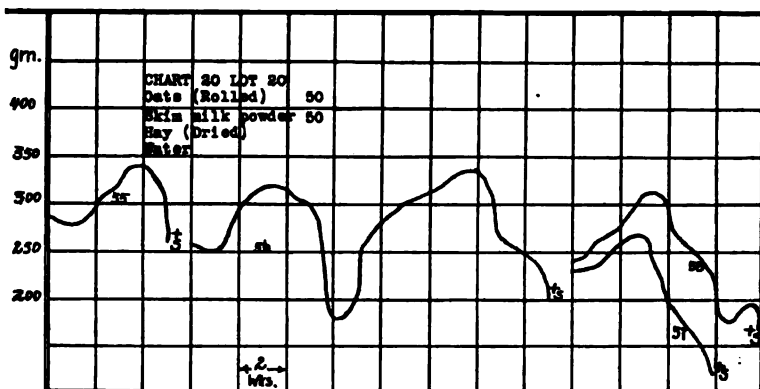


CHART 20. A still more liberal allowance of milk powder than was made with the lots whose curves are shown in Charts 18 and 19, failed to prevent the development of scurvy. The milk powder allowed per individual per day was equivalent to 75 to 90 cc. of fresh skim milk. Three of the animals rapidly succumbed to the disease, lasting but 5 to 7 weeks. Animal 56 developed scurvy as early as the other three in the lot, but in spite of the progress of the disease it started to regain in weight after the first period of 6 weeks, but finally died of scurvy at the end of 15 weeks.

NUTRITIONAL STUDIES ON THE GROWTH OF FROG LARVÆ (*RANA PIPPIENS*).

FIRST PAPER.

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PLATES 2 TO 5.

(Received for publication, April 21, 1919.)

A number of studies have been reported upon the growth and development of frog larvæ as influenced by feeding certain products *in toto*, such as glandular materials. As far as we are aware, however, no one has incorporated in the feeding of such larvæ conditions which were intended to control definitely the nutritive value of the diet in terms of protein, carbohydrate, fat, mineral salts, and the food accessories, hormones or vitamins. We have, therefore, attempted in this preliminary report to give a summary of our studies along this line with a view of supplementing it from time to time in the light of further findings.

Hopkins, Osborne and Mendel, McCollum and associates, and several others have made, during the last few years, valuable contributions, in their studies with rats and mice, to the nutritive value of synthetic food mixtures. They have shown that proteins, fats, salt mixtures, and vitamins each occupy a peculiar place in the physiological economy—not alone in respect to the amount present in diet but also in respect to the quality or kind of each nutrient.

It was thought by using this particular species of amphibians, namely frog larvæ (*Rana pipiens*) which have a characteristic life cycle—passing through the tadpole stage to the frog, that we might be able to determine some interesting facts regarding their nutrition with respect to size and degree of metamorphosis. One great advantage in using such larvæ in place of larger animals

is that one can work with large numbers and thereby be able to draw fairly definite conclusions. On the other hand, it is readily seen that serious objections may be raised in the making of practical tests. For example, if the larvæ are fed in large groups there is no way of measuring the food intake, or of preventing the more vigorous ones from taking the food from the weak, or of eating the tadpoles that die occasionally, and no means of controlling the water-soluble content of the diet if water is allowed to flow through the trays continuously.

EXPERIMENTAL.

The tadpole eggs were collected in the near vicinity and brought to the laboratory with every precaution to prevent injury, since in the late stages of segmentation the eggs are very delicate. Immediately after hatching, the tadpoles were grouped into colonies of 500 each, being placed in enamel-lined trays. Special attention was paid to the hygienic conditions of the surroundings. Thus, the ventilation of the room, which was used only for this work, was regulated; the bright light was eliminated; the trays were thoroughly cleaned at regular intervals; unused food was removed frequently to prevent the formation of toxic substance due to decomposition; and fresh water was supplied two to three times daily. The water used was from the city supply but it was first passed through a Hygeia filter which removed the suspended organic matter.

In order to obtain a permanent record of the progress of growth, photographs were taken each week of a selected group from each colony. The selection was governed by choosing from each tray an equal number of the largest, medium, and smallest specimens. Shadow photographs were made by subjecting the tadpoles to an instantaneous exposure of an arc light. From these photographic records, measurements were made of all the tadpoles, including body width, body length, and total length with tail.

Late in the experiment when the limb buds began to appear, microscopic examination of all the tadpoles in each tray was made. Following a definite scheme, each tadpole was classified according to its stage of development and from these data the

percentage distribution of the tadpoles was calculated for the groups that were on different diets. In addition to this an attempt was made to preserve every week representative specimens from every group and to make a histological study of the serial sections made from these specimens. The individual variations between the tadpoles proved to be too great a factor and for this reason the histological data will not be reported at this time. Text-fig. 1, page 333, illustrates the scheme followed in determining the various stages of development of the hind legs of the frog larvæ.

The feeding of the colonies or groups was done at specified times, always following the cleaning of the trays. The endeavor was to feed enough yet not too much, so as to avoid an excess of material remaining in the trays. This was governed in part by weighing out the food.

The synthetic diets were made up of various combinations of food substances: for protein, lactalbumin (1) and corn gluten (2) were used; cystine was also introduced as a supplement to corn gluten; for carbohydrate, purified starch or dextrin was used, and lactose, where Osborne and Mendel's protein-free milk (3) was incorporated; for fat, butter fat was employed as a carrier of fat-soluble A, lard was also used; for mineral salts, the McCollum Salt Mixture 185 was taken (4) except when protein-free milk was in the diet; for water-soluble B vitamins, either an alcohol extract of dried brewers' yeast, or protein-free milk was selected; and for another source of fat-soluble A, other than butter fat, we took an alcohol extract of linseed oil meal (5) which had first been treated with ether to remove the oil. Table I gives the composition of the diets fed.

DISCUSSION.

I. Effect of Diet on the Size of the Tadpole.

(A) *Influence of the Amount of Fat.*—In Table I, it will be seen that the rations or diets were arranged in two general classes, Groups I and II. The former was made up in accordance with the usual formula proposed by Osborne and Mendel; that is, these diets were high in fat, containing from 23 to 28 per cent of

328 Nutritional Studies on Frog Larvæ. I

TABLE I.
*Percentage Composition of the Diets.**

Diet No.	Foods in diet.		Diet No.	Foods in diet.	
		<i>per cent</i>			<i>per cent</i>
I ₁	Lactalbumin (protein).	10.0	II ₁	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Butter fat.	18.0		Butter fat.	5.0
	Lard.	10.0		Yeast extract (alcoholic.)	1.0
I ₂	Lactalbumin (protein).	10.0	II ₂	Lactalbumin (protein).	10.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	18.0		Butter fat.	5.0
	Lard.	10.0			
I ₃	Lactalbumin (protein).	10.0	II ₃	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Lard.	28.0		Yeast extract (alcoholic.)	1.0
I ₄	Lactalbumin (protein).	10.0	II ₄	Lactalbumin (protein).	10.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Lard.	28.0		Lard.	5.0
I ₇	Lactalbumin (protein).	10.0	II ₇	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Linseed meal extract (alcoholic).	5.0		Linseed meal extract (alcoholic).	5.0
	Lard.	23.0		Yeast extract (alcoholic).	1.0
				Lard.	5.0
I ₉	Lactalbumin (protein).	10.0	II ₉	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Yeast extract (alcoholic).	1.0		Yeast extract (alcoholic).	1.0
	Lard.	27.0		Lard.	5.0
III ₄	Corn gluten (protein).	10.0	III ₄	Corn gluten (protein).	10.0
	Salt mixture.†	3.7		Cystine.	0.2
	Butter fat.	5.0		Salt mixture.†	3.7
	Yeast extract (alcoholic).	1.0		Butter fat.	5.0
				Yeast extract (alcoholic).	1.0

TABLE I.—*Concluded.*

Diet No.	Foods in diet.		Diet No.	Foods in diet.	
		<i>per cent</i>			<i>per cent</i>
II _{1a}	Lactalbumin (protein).	30.0	II _{2a}	Lactalbumin (protein).	30.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1.0			
IV ₄	Lactalbumin (protein).	15.0	IV ₅	Lactalbumin (protein).	15.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1.0			
IV ₆	Lactalbumin (protein).	15.0	IV ₇	Lactalbumin (protein).	15.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Lard.	5.0		Yeast extract (alcoholic).	1.0
IV ₁₀	Beef tissue (protein).	15.8	V ₁	Lactalbumin (protein).	5.0
	Oats, rolled (protein).	1.9		Corn gluten (protein).	5.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1.0		Yeast extract (alcoholic).	1.0
III ₅	Lactalbumin (protein).	10.0	VI ₁	Lactalbumin (protein).	30.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Lard.	5.0
	Yeast extract (alcoholic).	1.0			

* In these rations purified starch was used in making up the total percentage, except in Ration III₅ where dextrin was employed.

† McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105. Salt Mixture 185.

either lard alone or a mixture of lard and butter fat. In group II, the McCollum formula was followed as to fat; *i.e.*, 5 per cent.

It will be seen from Fig. 1 that the tadpoles fed the high fat diet (I₁) were smaller than those that were fed the low fat diet (II₁). The periods referred to were 1 week apart. These permanent records were given from the time that differences began

to be evident. The diets in these two cases were complete in the sense that they were sufficiently well balanced as to all the essential nutrients needed to produce normal growth in young rats.

(B) *Influence of the Vitamines.*—Inasmuch as the low fat diet appeared to be best for the tadpole, it is obvious that any comparisons between groups with respect to their nutritive value should be made on this plane. If the diets from Groups II₁ and II₂ are compared, it will be seen that they differed in that the yeast vitamine was removed from the former and an equivalent amount of starch substituted for it. In Fig. 2, the control Group II₁ showed distinctly larger tadpoles than Group II₂, up to Period 10, when the differences were not nearly so marked. Whether this convergence was due to the fact that the diet for Group II₂ may have lacked some essential constituent which retarded a more rapid growth should be borne in mind. It is evident that the water-soluble B was essential up to a certain stage in the growth. Beyond this it may be that the tadpole was able to store enough of this form of vitamine in the tail to supply its needs, just as certain bacteria appear to need vitamines at the start but later they synthesize the vitamines themselves (6).

Ration II₂ was deficient in fat-soluble A vitamines but in other respects it corresponded to Ration II₁, lard being substituted for butter fat. From Fig. 3 it is evident that the tadpoles fed Ration II₁ grew better than those that lacked the fat-soluble A. After the eleventh period, however, the differences were too slight to be significant. In other words, the tadpoles seemed to need the fat-soluble accessory more in the early stages of growth, while later on there was an adaption, or a reserve supply furnished through the medium of the tail.

When both vitamines were withdrawn from the diet, the tadpoles did not grow so vigorously. Contrary to what we have found with rats, however, they did show a gradual rate of growth. This may have been due in part to their ability to draw on the reserve stored in the tail, for in each instance the tail length was much shorter than with the tadpoles fed Ration II₁. In all cases, though, the body length and body width were also less, so that there was a general reduction in body size. Figs. 4 and 5 illustrate this. In the former case, the diet was based on a 10 per cent protein plane and in the latter case on a 30 per cent protein

plane. The differences were more marked on the higher protein diet (Fig. 5).

(C) *Effect of the Amount of Protein.*—Osborne and Mendel (7) and Emmett and Luros (8) have been able to obtain normal growth with rats with 10 per cent of lactalbumin protein. Osborne and Mendel found that it required 50 per cent more casein and 90 per cent more edestin than lactalbumin to bring about normal growth in rats. Wheeler (9) in working with mice determined that the best results for growth were obtained when the casein was as high as 30 per cent. It might therefore be that while lactalbumin is one of the very best proteins for promoting growth, the quantity needed for tadpoles should be higher than for rats.

Fig. 6 shows that when the protein plane was 30 per cent (II_{1a}) the tadpoles grew more rapidly than when it was 10 per cent (II_1). In the former case the body width, length, and total length were greater than for Group II_1 . It should be stated that Group II_{1a} was obtained by subdividing Group II_1 in Period 9.

(D) *Effect of the Kind of Carbohydrate.*—In the large majority of the low fat diets, starch which had been purified by washing with hot 95 per cent alcohol was used as the sole source of carbohydrate. McCollum used dextrin in many of his rations and since the low fat diets were based essentially upon his formula, we incorporated dextrin in Ration III_6 to determine whether it was an essential factor and would accelerate growth. The comparative value of the two rations, II_1 and III_6 ,—the former starch and the latter dextrin—is shown in Fig. 7. There was practically no difference in the size of the two groups of tadpoles.

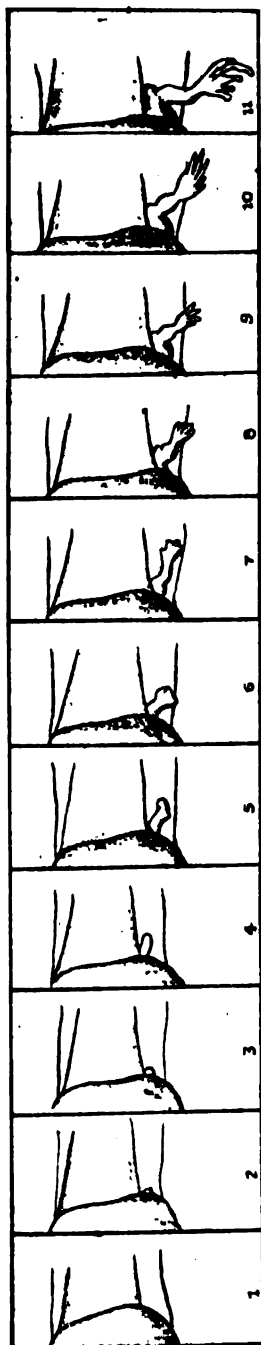
(E) *Value of Beef and Oat Diet.*—It is known that tadpoles will thrive on a diet of beef and oats. We therefore made up a control diet which was the same as Diet II_1 with the exception that we used 15.8 per cent of desiccated ether-extracted beef tissue protein and 1.9 per cent of ether-extracted rolled oats protein. Fig. 8 shows that we obtained our optimum growth with this ration. That this result was not due to the large amount of protein is evident, for when Fig 6, which shows tadpoles that were fed 30 per cent protein, is compared with Fig. 8, the beef-oats diet was the best although it contained 17.7 per cent protein.

To what this difference was due, we are not as yet able to state. However, in comparing the two rations II₁ and IV₁₀, it is evident that the beef tissue and rolled oats were the contributing factors; and that the presence of the butter fat, yeast extract, starch, and salt mixture in the diet did not inhibit growth. McCollum, Simmonds, and Parsons (10) claim that lactalbumin is an incomplete protein for growth. Emmett and Luros (8) found under certain conditions that they were not able to obtain good growth with lactalbumin but that the apparent trouble lay in the fact that this protein was either susceptible to toxic substances or else it needed the presence of a water-soluble vitamine other than the water-soluble B. When lactose or the protein-free milk of Osborne and Mendel was present, normal growth was obtained. It would appear that perhaps a similar condition of affairs took place with the tadpoles. This question is now being investigated.

II. Effect of Diet on Development of Hind Legs of Tadpoles.

Growth and development may bear only a limited relation to each other in tadpoles maintained in an artificial environment. To state that a specimen of certain measurements is in a corresponding stage of metamorphosis may, upon closer inspection, prove untrue. Individual variation in both respects has been found by us to be marked. Consequently the colonies should be fairly large and observation should be made on as many members as possible in order that the true average may be most nearly approached. Bearing this in mind, all the tadpoles in each of the groups were examined as to the stage of development. This, as was previously stated, was not carried out in the observations made for growth or size..

(A) *Influence of the Amount of Fat in the Diet.*—In Table II are presented the data giving the percentage distribution of the tadpoles in each group with respect to the stage of development of the hind legs. These data, for two of the experimental periods (Nos. 12 and 14), near the close of the series of tests, are arranged chiefly with reference to the amount of fat in the diet, and also the kind of vitamine that is present. The detailed percentage composition of the diets is given in Table I. It is to be noted



TEXT-FIG. 1. The above drawings illustrate the scheme followed in determining the classification of the frog larvae with respect to the development of the hind legs—as used in Tables II, III, and IV.

334 Nutritional Studies on Frog Larvæ. I

TABLE II.

Influence of Amount of Fat upon Development of Hind Legs of Rana pipiens Larvæ.

Percentage Distribution within Groups.

Group.	Diet.		Experimental period.	Stages of development of hind legs.*										
	Class of vitamins.	Fat.		1	2	3	4	5	6	7	8	9	10	11
		per cent												
I ₁	Water- and fat-soluble.	28†	12	11.1	62.9	22.9	6.3	—	—	—	—	—	—	—
I ₁		28†	14	—	45.4	36.3	18.2	—	—	—	—	—	—	—
II ₁		5‡	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II ₁		5‡	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9	—
I ₇	Water- and fat-soluble.	23§	12	8.1	43.9	40.0	8.1	—	—	—	—	—	—	—
I ₇		23§	14	6.6	50.4	32.4	10.4	—	—	—	—	—	—	—
II ₇		5§	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	0.5
II ₇		5§	14	8.6	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
I ₂	Fat-soluble.	28†	12	31.3	43.4	20.2	5.1	—	—	—	—	—	—	—
I ₂		28†	14	28.3	35.8	25.0	10.9	—	—	—	—	—	—	—
II ₂		5‡	12	9.4	40.5	36.5	12.2	1.4	—	—	—	—	—	—
II ₂		5‡	14	—	20.3	53.0	17.2	7.8	1.6	—	—	—	—	—
I ₃	Water-soluble.	28§	12	11.4	56.1	26.5	5.3	0.8	—	—	—	—	—	—
I ₃		28§	14	13.7	34.4	41.0	10.7	—	—	—	—	—	—	—
II ₃		Nore.	12	1.5	12.1	34.8	24.2	15.1	9.1	—	1.5	—	—	1.5
II ₃		"	14	3.3	13.1	18.0	41.0	8.2	3.3	8.2	3.3	1.6	—	—
I ₉	Water-soluble.	27§	12	—	65.3	26.5	8.2	—	—	—	—	—	—	—
I ₉		27§	14	—	68.9	17.3	13.8	—	—	—	—	—	—	—
II ₉		5§	12	23.9	51.4	17.4	6.5	0.7	—	—	—	—	—	—
II ₉		5§	14	17.6	30.4	39.2	10.8	1.9	—	—	—	—	—	—
I ₄	None.	28§	12	11.2	46.5	33.6	8.6	—	—	—	—	—	—	—
I ₄		28§	14	10.2	35.1	32.4	21.3	0.9	—	—	—	—	—	—
II ₄		5§	12	17.0	55.2	19.5	7.3	—	—	—	—	—	—	—
II ₄		5§	14	16.6	39.0	28.0	16.6	—	—	—	—	—	—	—

* See Text-fig. 1, p. 333.

† 18 per cent butter fat and 10 per cent lard.

‡ Butter fat.

§ Lard

that the group number and diet number correspond. Thus, in Table II, Group I₁ was fed Diet I₁.

If Group I₁ (28 per cent fat) is compared with Group II₁ (5 per cent fat) it is seen, when both the water-soluble B and fat-soluble A accessory substances were present, that the tadpoles fed the low fat diet developed much better. In Groups I₇ and II₇, the fat-soluble A was supplied by an alcoholic extract of linseed meal, whereas it was furnished in the two preceding groups by butter fat. The difference between these two groups was clearly in favor of the low fat diet. With Groups II₁ and II₇, we have a comparison of two kinds of fat, butter fat and lard, and it appears that 5 per cent of either was permissible for fair development. Whether a smaller amount would have been much better is being tried out.

When the diet lacked the water-soluble B vitamine and contained the fat-soluble A in the form of butter fat, as shown in Groups I₂ and II₂, the low fat diet produced the best results. The differences were not so marked, however, as in the cases of Diets I₁, I₇, II₁, and II₇.

Groups I₃ and II₃ were fed diets which were deficient in fat-soluble A. In the case of the latter group no fat was used. It will be seen that this group was far in advance of the high fat-fed colony. In fact, there is such a marked contrast that the question immediately arises as to what extent Group II₁ would have developed if we had used some other source of fat-soluble A than a fat, as butter fat, and what results would have been obtained if the lard had been omitted from Diet II₇. In Groups I₃ and II₃, this point is partially answered since the diet for II₃ differed only from that of II₂ in having 5 per cent lard added to it and in having a corresponding decrease in starch. It would appear in comparing Groups II₃ and II₂ that the fat had a marked inhibiting effect. With respect to Groups I₃ and II₃, which contained 27 and 5 per cent of lard respectively, there was only a slight difference in favor of the lower fat diet.

When both vitamines were absent, as in the Groups I₄ and II₄, the high fat diet appeared to produce somewhat better results than the low fat diet.

It is evident from these data that the amount of fat in the diet had an appreciable influence upon the development of the tad-

336 Nutritional Studies on Frog Larvæ. I

TABLE III.

*Influence of Vitamines upon Development of Hind Legs in Rana pipiens Larvæ.
Percentage Distribution within Groups.*

Group.	Diet.		Experimental period.	Stages of development of hind legs.*										
	Class of vitamines.	Fat.		1	2	3	4	5	6	7	8	9	10	11
		per cent												
I ₁	Water- and fat-soluble.	28†	12	11.1	62.9	22.9	6.3	—						
I ₁	" " "	28†	14	—	45.4	36.4	18.2	—						
I ₇	" " "	23‡	12	8.1	43.9	40.0	8.1	—						
I ₇	" " "	23‡	14	6.7	50.5	32.4	10.4	—						
I ₂	Fat-soluble.	28†	12	31.3	43.4	20.2	5.1	—						
I ₂	"	28†	14	28.2	35.9	25.0	10.9	—						
I ₃	Water-soluble.	28†	12	11.4	56.1	26.5	5.3	0.8						
I ₃	"	28†	14	13.7	34.3	41.2	10.8	—						
I ₉	"	28†	12	—	65.3	26.5	8.2	—						
I ₉	"	28†	14	—	68.9	17.3	13.8	—						
I ₄	None.	28†	12	11.2	46.5	33.6	8.6	—						
I ₄	"	28†	14	10.2	35.2	32.4	21.3	0.9						
II ₁	Water- and fat-soluble.	5§	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II ₁	" " "	5§	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9	—
II ₇	" " "	5‡	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	0.5
II ₇	" " "	5‡	14	8.6	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
II ₂	Fat-soluble.	5§	12	9.4	40.5	36.5	12.2	1.4	—	—	—	—	—	—
II ₂	"	5§	14	—	20.3	53.1	17.2	7.8	1.6	—	—	—	—	—
II ₃	Water-soluble.	None.	12	1.5	12.1	34.8	24.2	15.1	9.1	—	1.5	—	—	1.5
II ₃	"	"	14	3.3	13.1	18.0	41.0	8.2	3.3	8.2	3.3	1.6	—	—
II ₉	"	5‡	12	23.9	51.4	17.4	6.5	0.7	—	—	—	—	—	—
II ₉	"	5‡	14	17.6	30.4	39.2	10.8	1.9	—	—	—	—	—	—
II ₄	None.	5‡	12	17.0	53.2	19.5	7.3	—	—	—	—	—	—	—
II ₄	"	5‡	14	16.6	39.0	27.8	16.6	—	—	—	—	—	—	—

* See Text-fig. 1, p. 333.

† 18 per cent butter fat and 10 per cent lard.

‡ Lard.

§ Butter fat.

pole; *i.e.*, the higher the fat content, the lower the stage of development.

(B) *Influence of Vitamines.*—In Table III, the data for the percentage distribution of the tadpoles within the groups are arranged with respect to the presence or absence of vitamins in the diet. The upper half of the table gives the results for the high fat diets. It would appear that the excess of fat was such a large factor that there was practically no difference between the groups.

In regard to the low fat diet Group II₁ was somewhat further advanced than Group II₇. This may have been due to the lard or to the fact that the fat-soluble A accessory was supplied either qualitatively or quantitatively to a lesser degree than in II₁. When Groups II₁, II₂, and II₃ are compared, the withdrawal of either the water-soluble B (II₂) or of the fat-soluble A (II₃) resulted in a distinct inhibition or retardation in the development of the tadpoles. In the case of Group II₃, two factors seemed to have played a part, the lack of both fat-soluble A and of any fat. As a result, this group developed at practically the same rate as Groups II₁ and II₇. This would tend to suggest that the water-soluble B vitamin was more essential than the fat-soluble A, or that the tadpoles had a reserve supply of this latter accessory stored in the tail, upon which they could draw. When both vitamins were removed from the diet, however, it appears from Group II₄, that there was little or no reserve vitamin supply available, for these tadpoles developed more slowly than any of the other groups.

The data indicate that both vitamins are essential to the development of the tadpole; that when fat was present to the extent of 5 per cent and the fat-soluble A was absent, there was a marked suppression, but if there was no fat present, the rate of development appeared to be normal when compared with the groups fed the control diets (II₁ and II₇). When the water-soluble B vitamin was absent the cessation of the development of the tadpoles was very noticeable.

(C) *Influence of the Amount and Kind of Protein.*—The data in Table IV are so arranged that the values can be compared with respect to the amount of protein. There is sufficient information given to indicate the vitamins and also the amount and kind

TABLE IV.
Influence of Amount and Kind of Protein upon Development of Hind Legs of *Rana pipiens* Larvæ. Percentage Distribution within Groups.

Group.	Diet.		Experi- mental period.	Stages of development of hind legs.*												
	Protein.			Class of vitamins.	Fat.											
	Kind.	per cent														
						1	2	3	4	5	6	7	8	9	10	11
II ₁	Lactalbumin.	10	Water- and fat soluble.	5†	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II ₁	"	10	"	5†	14	—	14.6	34.7	29.5	7.4	2.4	—	4.9	2.4	4.9	—
IV ₄	"	15	"	5†	12	5.1	21.4	21.4	26.6	15.2	3.8	2.5	1.3	—	2.5	—
IV ₄	"	15	"	5†	14	—	32.5	47.5	5.0	2.5	7.5	2.5	2.5	—	—	—
III ₇	"	30	"	5†	12	2.8	13.9	36.1	22.2	13.9	—	—	11.1	—	—	—
III ₇	"	30	"	5†	14	—	3.4	17.2	37.9	10.4	10.4	6.4	—	3.4	3.4	6.9
II ₇	"	10	"	5†	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	—
II ₇	"	10	"	5†	14	8.4	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
III ₆ §	"	10	"	5†	12	0.8	23.7	38.1	17.9	6.8	6.0	1.7	0.8	2.5	—	—
III ₆ §	"	10	"	5†	14	2.3	25.5	38.9	15.0	5.5	4.0	5.5	1.6	0.8	—	—
II ₂	Lactalbumin.	10	Fat-soluble.	5†	12	9.4	40.5	36.5	12.2	1.4	—	—	—	—	—	—
II ₂	"	10	"	5†	14	—	20.3	53.1	17.2	7.8	1.6	—	—	—	—	—
IV ₆	"	15	"	5†	12	9.9	43.2	36.0	10.8	—	—	—	—	—	—	—
IV ₆	"	15	"	5†	14	3.8	22.6	47.2	22.8	—	1.9	—	—	—	—	—
II _{2a}	"	30	"	5†	12	27.3	34.5	21.8	12.7	1.8	1.8	—	—	—	—	—
II _{2a}	"	30	"	5†	14	36.0	12.0	18.0	20.6	6.0	2.0	—	—	—	—	—

II ₂	Lactalbumin.	10	Water-soluble.	None.	12	1.5	12.1	134.8	24.2	15.1	9.1	—	1.5	—	1.5
II ₃	"	10	"	"	14	3.3	13.1	118.0	41.0	8.2	3.3	8.2	3.3	1.6	—
IV ₆	"	15	"	"	12	3.1	34.6	22.3	23.8	13.1	0.1	2.3	—	—	—
IV ₈	"	15	"	"	14	8.3	28.8	26.7	24.2	6.7	5.3	—	—	—	—
VI ₁	"	30	"	"	12	3.0	19.9	28.8	38.0	7.6	3.0	—	—	—	—
VI ₁	"	30	"	"	14	8.3	1.7	30.0	35.0	16.6	5.0	3.3	—	—	—
II ₆	Lactalbumin.	10	None.	5†	12	17.0	56.2	19.5	7.3	—	—	—	—	—	—
II ₆	"	10	"	5†	14	16.6	39.0	27.8	16.6	—	—	—	—	—	—
IV ₆	"	15	"	5†	12	5.1	50.0	31.6	12.5	0.7	—	—	—	—	—
IV ₆	"	15	"	5†	14	10.3	20.6	41.1	16.9	1.8	—	—	—	—	—
VI ₁	"	30	"	5†	12	11.9	45.2	23.8	19.0	—	—	—	—	—	—
VI ₁	"	30	"	5†	14	23.6	20.6	38.2	15.0	2.9	—	—	—	—	—
IV ₁₀	Beef and oat.	17.7	Water-and fat-soluble.	5†	12	4.6	18.9	24.5	26.5	11.8	6.6	2.5	2.5	—	2.0
IV ₁₀	"	17.7	"	5†	14	4.3	16.9	21.8	22.5	14.1	7.6	4.9	0.5	2.1	13.2
II ₁	Lactalbumin.	10	"	5†	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1
II ₁	"	10	"	5†	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9
V ₁	Lactalbumin, corn gluten. Ratio 1:1.	10	Water-and fat-soluble.	5†	14	2.4	23.8	42.8	21.4	4.8	1.2	2.4	—	1.2	—
III ₁	Corn gluten, cystine.	10	Water-and fat-soluble.	5†	12	12.2	49.0	30.0	2.8	2.8	3.0	—	—	—	—
III ₁	"	10	"	5†	14	10.5	41.1	32.6	10.5	3.2	2.1	—	—	—	—
III ₁	Corn gluten.	10	Water-and fat-soluble.	5†	12	13.6	41.9	40.7	3.7	—	—	—	—	—	—
III ₁	"	10	"	5†	14	5.6	45.0	39.4	8.4	1.4	—	—	—	—	—

* See Text-fig. 1, p. 333.

† Butter fat.

‡ Lard.

§ Same as II, except dextrin used for starch.

of fat that were present. Groups II₁, IV₄, III₇, and II₇ were fed lactalbumin in amounts of 10, 15, 30, and 10 per cent respectively with both vitamins and 5 per cent of fat. The results indicate that the amount of protein had no influence on the rate of development. When the water-soluble B accessory was absent and the percentage of lactalbumin varied from 10 to 30 per cent (Groups II₂, IV₅, and II_{2a}), the 30 per cent protein diet (II_{2a}) was somewhat the best. On the other hand, with no fat-soluble A and no fat in the diets (Groups II₃, IV₈, and VI₁), the results were slightly in favor of the 10 per cent protein diet (II₃). The non-vitamine groups (II₄, IV₆, and VI₂) show that the 10 per cent protein plane (II₄) was too low, while there was no difference between the 15 and 30 per cent diets.

To sum up, it would seem that in the main the amount of protein, whether 10, 15, or 30 per cent of lactalbumin, had comparatively little to do with the rate of development of the hind legs. There were some variations within the classes of vitamins that showed differences, but taken as a whole, one is hardly justified in placing too much emphasis upon these differences when the data for the corresponding periods are compared. This finding compares with rats where 10 per cent lactalbumin produces normal growth when properly supplemented.

If Group IV₁₀, fed the beef-oat protein diet, is compared with Group II₁, the percentage distribution is found to be in favor of IV₁₀ when Period 14 is considered, but here again we are of the opinion that on the average it would be fairer to assume perhaps that there was not much difference, although when Fig. 8 is borne in mind we are almost forced to conclude that Ration IV₁₀ was superior to Ration II₁. Here again it would seem that the amount of protein had little to do with the differences when we compare the data with that for Diet III₇. Furthermore, it does not seem to be a matter of vitamins or fat, for these are present in each diet. The beef or oats seemed to have carried other accessories or else the quality of protein was better adapted to the tadpole.

That lactalbumin was at least a fairly good protein for growth and development is shown from the results obtained by comparing it with a poor protein like corn gluten, which does not produce growth in rats (1, 2). Group III₅ was fed the same diet

as II₁ except that corn gluten was substituted for lactalbumin. There was a distinct difference between the two in favor of the lactalbumin. When corn gluten was supplemented with an equal amount of lactalbumin protein (Ration V₁), it is seen that the lactalbumin was able to stimulate development to some extent but not as much as when it was used as the sole protein.

Osborne and Mendel (1) found that cystine was able to supplement a low (9 per cent) casein diet and produce the same rate of growth as a 15 per cent casein ration, without cystine. In adding cystine to a 10 per cent corn gluten diet (Group III₄), we obtained slightly better results than when no cystine was present.

From the discussion on the amount and quality of protein in the diet, it would appear that the amount of protein, from 10 to 30 per cent, had little or no influence upon the development of the tadpole, but that the quality of protein was an important factor.

(D) *Influence of Kind of Carbohydrate.*—In Table IV, it is possible to make a comparison of the value of dextrin and starch, Groups III₆ and II₁, respectively. It is seen that there was no difference between the two.

CONCLUSIONS.

From the data herein reported upon the size and development of the frog larvæ (*Rana pipiens*), the following tentative conclusions are made, subject to revision in the light of studies which are being started for this season, and which will be carried out with a special effort to control more carefully the matter of temperature of water and the possibilities of dominance of one individual over the other.

1. A large amount of fat in the diet was very injurious to both growth (body size) and development of hind legs. Apparently the lower the percentage of fat the better the tadpoles thrived.

2. Vitamines—the water-soluble B and fat-soluble A types—appeared to be necessary for normal growth and development. The lack of the water-soluble B type was possibly more apparent than that of the fat-soluble A. When no vitamins were present, growth and development were distinctly retarded.

342 Nutritional Studies on Frog Larvæ. I

3. The amount of protein incorporated seemed to play a minor part in the development of hind legs when the per cent of lactalbumin ranged from 10 to 30—whether both “vitamines,” one, or none, were present. On the other hand, the high protein fed tadpoles were largest (size) when both vitamins were in the diet.

4. The quality of protein was a factor which showed clearly that it should be borne in mind. Thus, lactalbumin, and beef and oats protein when incorporated in an otherwise complete ration brought about a good rate of development, while corn gluten protein gave poor results.

5 When dextrin was substituted for starch it had no effect either on growth or development.

6. The results taken as a whole show definitely that it is possible to adjust the diet so as to alter the size of the tadpole and the rate of metamorphosis, in respect to variations in such nutrients as fat, kind of protein, and vitamins. It should be borne in mind that other factors play an important part, such as temperature and food control. Too low temperatures tend to make the tadpoles sluggish, and too high temperatures produce abnormal changes which may result in death. The tendency to consume their dead makes it difficult at times to adjust the diet absolutely.

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EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Photographic reproductions of tadpoles, showing the relative size as influenced by a high (I₁) and a low (II₁) fat diet.

FIG. 2. The diets given were both on the low fat plane. Diet II₁ contained the water-soluble B and the fat-soluble A vitamins, while Diet II₂ was lacking in the water-soluble type.

PLATE 3.

FIG. 3. Both diets contained the low percentage of fat. Diet II₁ was considered to be complete for normal growth and Diet II₂ was deficient in the fat-soluble A vitamin.

FIG. 4. Diet II₁ was compared with Diet II₂. The latter was lacking in both the water-soluble B and the fat-soluble A vitamins. These diets contained 10 per cent protein.

PLATE 4.

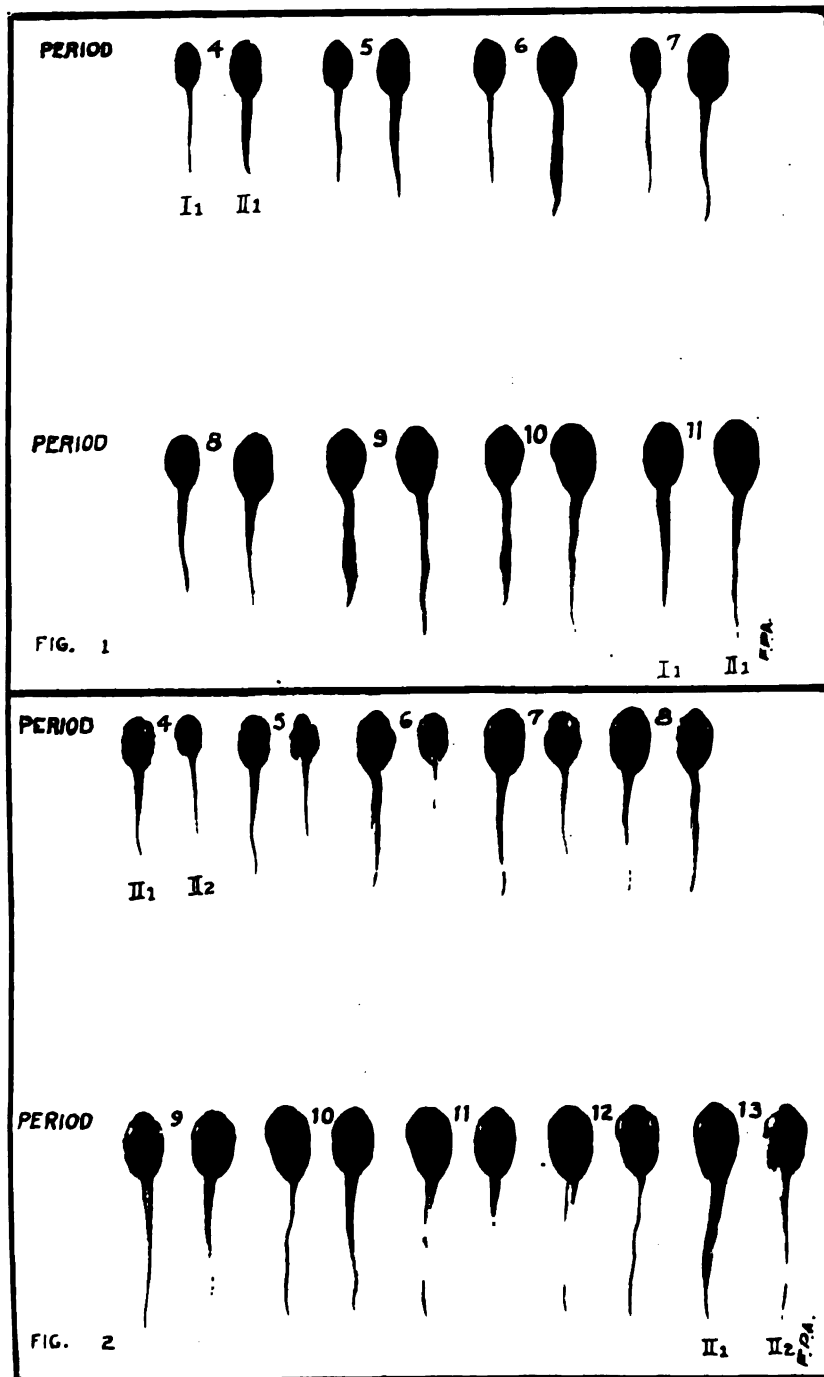
FIG. 5. The diets fed in this case were: complete (II_{1a}), and deficient in the vitamins (VI₁). They contained 30 per cent protein instead of 10 per cent as shown in Plate 3, Fig. 4.

FIG. 6. Both diets were complete. Diet II₁ had 10 per cent protein and Diet II_{2a}, 30 per cent protein.

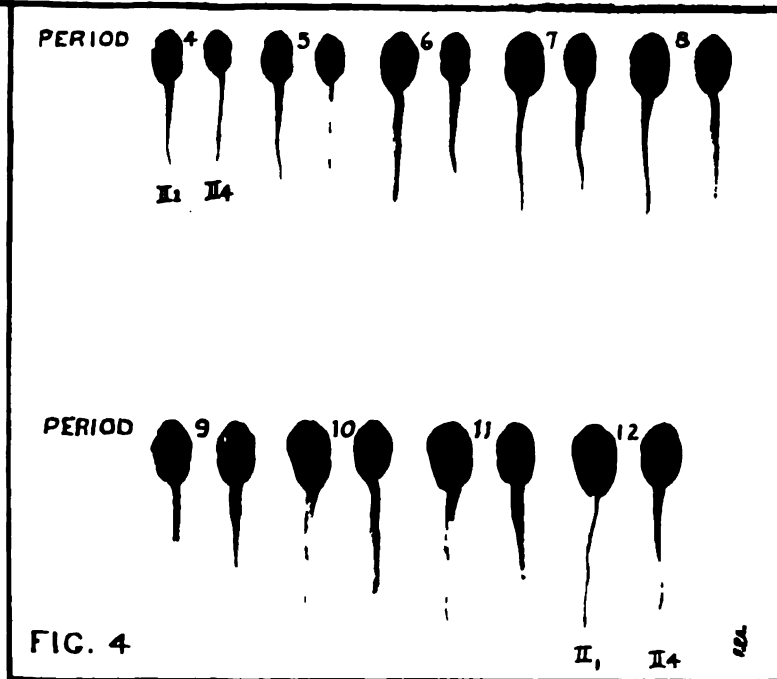
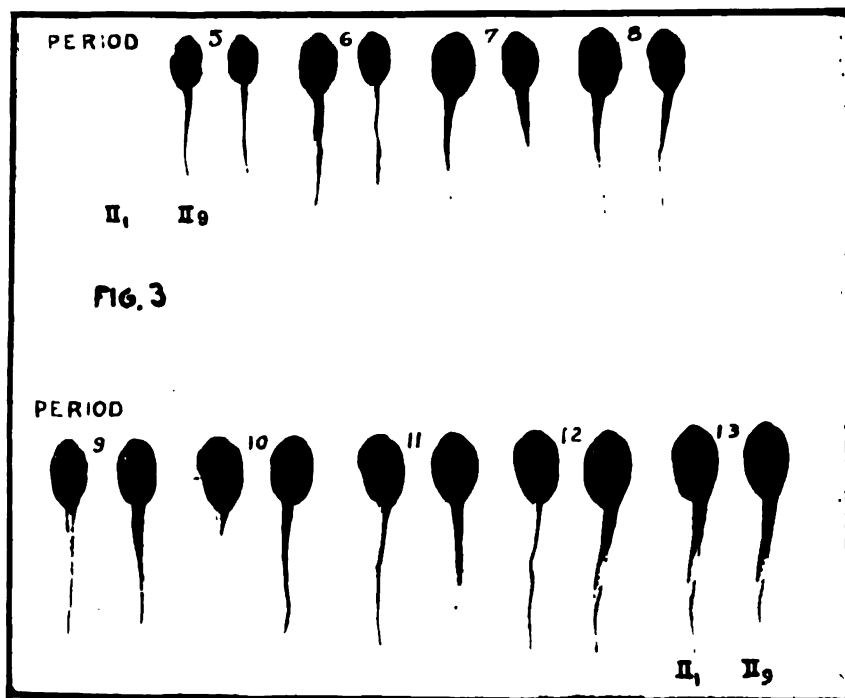
PLATE 5.

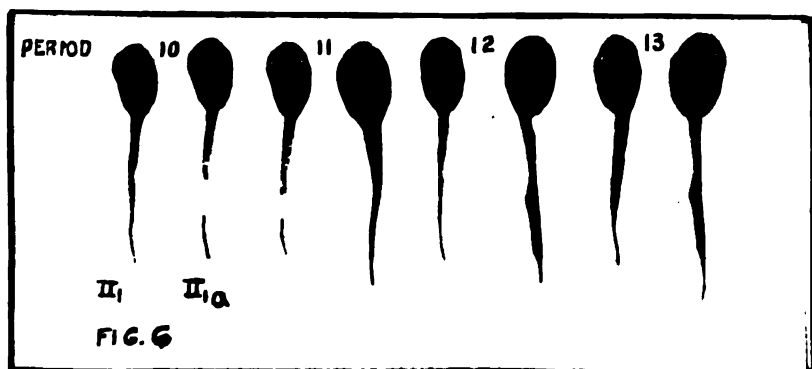
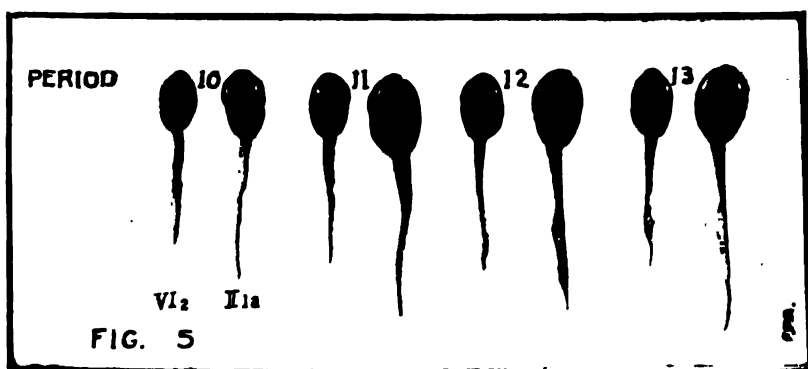
FIG. 7. The two diets differed in the kind of carbohydrate used. Diet II₁ contained starch and Diet III₂ an equivalent quantity of dextrin.

FIG. 8. Diet IV₁₀ differed from Diet II₁ in having the protein derived from desiccated beef tissue and rolled oats which had been extracted with ether. The former diet contained 17.7 per cent protein and the latter 10 per cent protein. These photographs should also be compared with those in Plate 4, Fig. 6, where Diet II_{1a} contained 30 per cent protein.

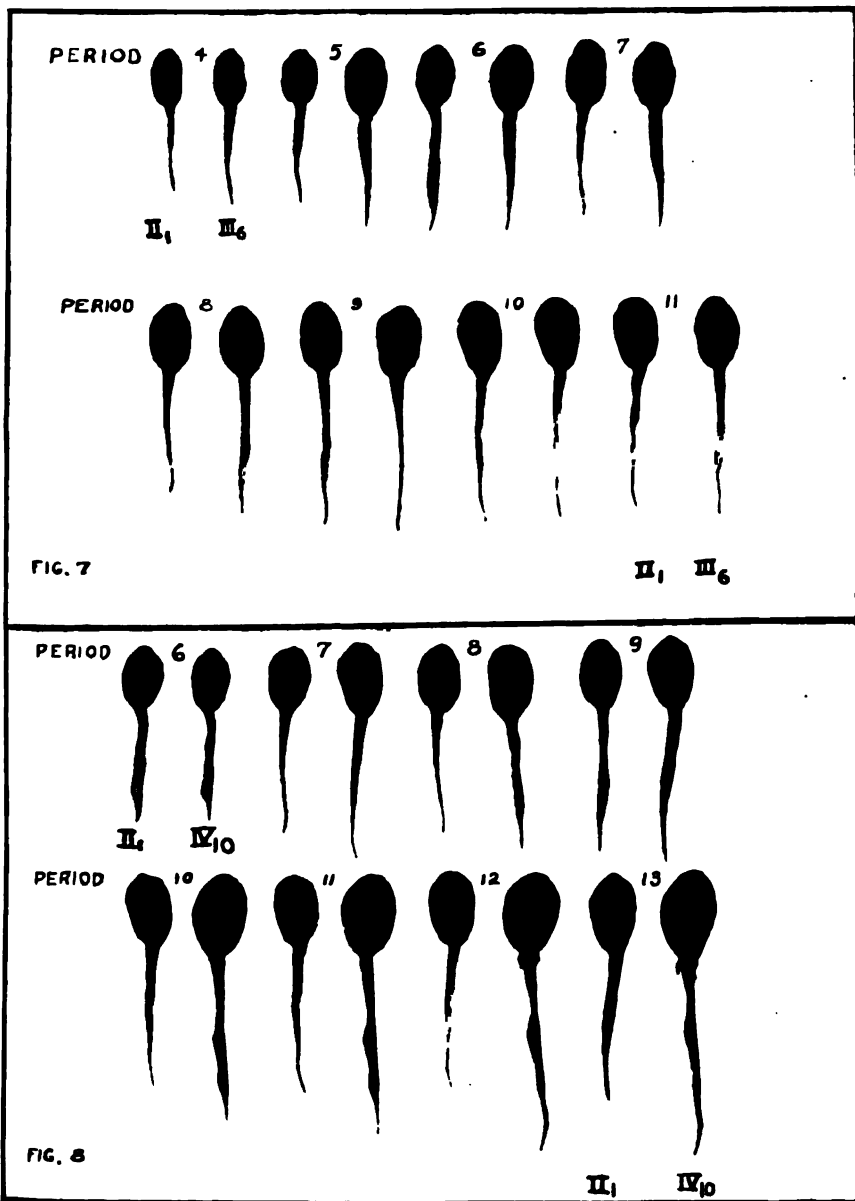


(Emmett and Allen: Nutritional studies on frog larva.)





(Emmett and Allen: Nutritional studies on frog larvae.)



(Emmett and Allen: Nutritional studies on frog larvae.)

THE AGE AT WHICH TRYPSINOGEN APPEARS IN THE FETAL PANCREAS.

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INTRODUCTION.

The value of an investigation of the passive physiology of the embryo mammal is apparent to workers in many scientific fields. The physicochemical relationship which the factors concerned in embryonic growth bear to the growth and nutrition of the adult has been brought to light by the work of Mendel and his collaborators, Mitchell (1), Saiki (2), and Leavenworth (3). In this work the demonstration of the presence or absence of many vital products, including certain enzymes, in pig embryos of various ages, played an important part. Buxton and Shaffer (4) investigated the occurrence of certain enzymes in very young pig embryos, with special reference to a purely pathological problem. Their goal was the disproval of the theory of the fetal origin of certain mammalian neoplasms by contrasting the enzymes found in these growths with those found in the embryos. Jones and Austrian (5), working toward a closer differentiation of the nucleoprotein-digesting ferments, used the liver of the pig embryo of various ages to demonstrate the separate appearance of the individual ferments.

The fixation of a comparatively definite point in the growth of an animal, below which no appreciable amount of a proteolytic zymogen can be demonstrated in the pancreas, will lay the foundation for a large amount of work, anatomical, chemical, and physiological, and will give us a more complete understanding of the nature of this subject. It has been the sole object of this work to establish the time when the presence of a proteolytic zymogen can first be demonstrated in the pig fetus.

A review of the literature reveals no specific work with the embryonic pancreas having for its objective the determination of its proteolytic power, but the work of the men above mentioned offers some clue as to the time of onset of some of the special glandular functions. Buxton and Shaffer (4) found amylase and lipase present in extracts of whole pig embryos of 10 mm. size, oxidases at 26 mm., and proteolytic enzymes appeared to be variable, occurring between 26 mm. and 85 mm. Jones and Austrian (5) could first demonstrate adenase in the liver of fetal pigs of 150 mm. size, but guanase not until after birth. Catalases were shown to be present in the liver, lung, muscle, and brain as early as 65 mm., by Mendel and Leavenworth (3); aldehydase, as early as 80 mm. was found by Jacoby (6) in whole embryo preparations; while Batelli and Stern (7) in similar preparations found lipase in very young animals. Mendel and Mitchell (1) succeeded in obtaining lactase and maltase from extracts of intestinal mucosa of fetuses as young as 75 mm.

In the instances quoted above, the fetus of the domestic pig was employed, for the same reasons, presumably, as in the present instance; namely (1) the easy access to many litters of all ages at the slaughter houses, and (2) their larger size as compared with other accessible fetuses, enabling the dissection of the pancreas with facility. In all succeeding statements reference to the fetuses will be made in terms of millimeters, which represent, in the animals handled by myself, the linear distance from the base of the tail to the crown of the head when the animal is lying in its natural curvature on a flat surface. With reference to other work, although the method of measurement was not included in the articles, we may assume that approximately the same system was employed. Since from 4 to 12 fetuses were dissected in the litters employed, it is to be expected that in the larger animals (those over 100 mm.), a variation of a few mm. in the fetuses of the same litter was frequently found, and the average of the lengths was therefore taken for the record. Such a variation in individuals from a litter whose average length was less than 100 mm. caused me to suspect that the irregular animals were abnormal and they were therefore rejected for the preparation of extract.

Although some of the above results are at variance, a striking feature of a general survey is that the special glandular functions, particularly in the organs arising in connection with the intestinal tract, come into a measurable degree of development between 65 and 85 mm. The result of the work herein given serves to strengthen this generalization, and likewise harmonizes with the work of Corner (8) on the anatomy of the fetal pig's pancreas in various stages of development. He found that the practically homogenous plexus constituted by the anastomosing embryonic ducts, gives way to a coordinated tree-like pattern, constituted by ducts of various orders, the process being completed between the ages of 60 and 85 mm. He likewise found that the differentiation of the glandular units is begun about the same period. It is consequently interesting that the production of intracellular zymogen by the cells of this organ is coincident with the development of a mechanism for the output of these products.

EXPERIMENTAL.

It is unnecessary to describe the evolution of the method as described below, except for the statement in passing that the technique employed by Buxton and Shaffer (4) in their work on proteolytic enzymes, was found to be inadequate for any constant quantitative or even qualitative estimate of the digestive action of a pancreatic extract, and was therefore abandoned after several trials. In brief the criterion of this method was the appearance of a clear ring about a drop of a proteolytic solution on a milk-agar Petri plate, indicating the digestion of the casein in the neighborhood of the ferment.

In the adoption of a technique three factors had to be considered: (1) obtaining a potent extract of the pancreatic tissue in a utilizable amount from a series of animals of successive ages; (2) the exclusion of all factors tending to produce any effect on the final readings which would mask the results obtained from the action of the extract; and (3) the accurate measurement of the digestive action of this extract.

To insure the first and second conditions, the following technique was employed. The pancreas was dissected out of each fetus of the individual litters, taking care in the procedure to

prevent the bursting of the stomach or the inclusion of an appreciable amount of blood. All the glands from the same litter were then placed in stoppered bottles and allowed to stand at room temperature for 24 hours. This lapse of time served to encourage bacterial growth whose action, according to Mellanby and Wooley (9), transforms a maximum amount of trypsinogen into trypsin within this period of time. The bacteria which are supposed to bring about this activation are *Bacillus subtilis*, *Bacillus coli*, and Moeller's dung bacillus which are commonly present in the circulating air. In contrast to this theory, according to Morse (10) and Bradley (11), zymogens will be converted into enzymes in sterile surroundings, and to support this theory Nelson and Long (12) prepared proteolytic pancreatic extracts from freshly triturated gland, claiming that the extraneous tissue included contained sufficient enterokinase to activate a maximum amount of the extract. For the purpose of this work the interval of 24 hours in time gave a maximum amount of proteolytic enzyme.

The amount of pancreatic connective tissue in ratio to the actual parenchyma, when collected in the entire gland mass, is one of the variable factors tending to make the proportional amount of functional enzyme appear less in the tabulated results of the younger embryos than really exists. The second disturbing factor which is, however, negligible for the series of litters done simultaneously, but which causes a variation of some magnitude in the results of series done on different days, is the unknown degree to which activation has proceeded. Because of this latter factor the results of the experiments have been placed in three separate columns, as noted in Table I, each column consisting of the series of litters employed simultaneously.

After the activation of the zymogen has taken place a glycerol extract of the gland was prepared in slightly basic medium, by triturating it with washed quartz sand in the proportion of 1 gm. of gland to 5 cc. of glycerol and 5 cc. of 0.5 N Na_2CO_3 . Vernon (13) has shown that glycerol pancreatic extracts are more stable than alcoholic extracts while no other extractive is as effective as either of these two.

The trituration was continued until a smooth milk-like suspension of the gland in the fluid was obtained and 0.2 cc. of

this freshly prepared material was pipetted into each of four clean dry glass tubes of about 5 cm. length and 4 mm. inside diameter. To each tube was then added 2 cc. of 0.5 N Na_2CO_3 , as this amount furnished approximately the concentration of OH-ions, found by Vernon (13) to give the optimum reaction for the tryptic digestion of protein. This optimum alkalinity was found by Vernon (13) to be about $\frac{1}{1.260}$ N OH^- and these results were also approximately obtained by Michaelis and Davidsohn (14) and later by Long and Hull (15), while the explanation for the necessity of this optimum alkalinity was given by Robertson and Schmidt (16).

As a relatively constant and easily accessible source of water-soluble, digestible protein, minced coagulated egg white, which had been soaked for 24 hours and then partially desiccated, was added in 0.2 gm. masses to the tubes to serve as the substrate for the ferment. To prevent the action of proteolytic bacteria, it was necessary to employ a bactericide. The choice of a proper antiseptic was difficult in that it must not be toxic to the ferment like the phenols or the salts of the heavy metals (mercury, copper, arsenic, or silver), nor a protein coagulant like the latter mentioned salts, nor must it be volatile like chloroform or toluene, since the tubes are to be sealed and heated. The cyanides and fluorides are the salts approximating the necessary conditions most closely, although both are markedly toxic. However, the fluorides are the less toxic of the two and Vernon (13) has shown that NaF is less so than NH_4F , a concentration of 0.5 per cent NaF sufficing to inhibit bacterial growth completely without materially altering the speed of the protein hydrolysis. On the basis of this work I added 0.26 cc. of 4 per cent NaF to each of the tubes containing the extract and protein, to obtain the optimum concentration.

Holding the tubes at a level above the contained fluid in order to insure that the material would remain cool, the open ends were drawn out and sealed in a flame to guard against the possible factor of evaporation occurring irregularly during the processes of sealing.

Of the four identical tubes, two were heated in the water bath to a temperature of 97–100°C. for a period of 5 minutes, which procedure has been found by Ohta (17), de Souza (18), and

many other investigators to destroy completely the potency of trypsin. We now have four tubes apparently identical as to their contents except that two contain an active ferment, if such existed in the original pancreas, while the other two tubes are entirely inactive. They were now incubated at 38°C. for 24 hours, this being approximately the optimum temperature and time for tryptic hydrolysis of protein. After incubation the tubes were centrifuged until the supernatant fluid was freed from all suspended matter. If ferment had been present in the unheated tubes, the product of its hydrolysis would now be included in the fluid contents of the tubes.

The third condition, as mentioned in the beginning of this section, namely, the accurate mensuration of the digestive action of the pancreatic extract, must now be fulfilled. This could be accomplished through the measurement of the amount of protein digested in the tubes containing the enzyme, and several methods were available. The biuret test and Kjeldahl estimation of nitrogen have been employed by Vernon (13), the polariscopic method by Koelker (19), the colorimetric method by Harding and MacLean (20), and the Van Slyke method by Long and other investigators (21). Owing to the extremely small amount of utilizable fluid, I adopted, at the suggestion of Professor Robertson (22), the refractometric method as first developed by Reiss (23) for determining the total protein in blood serum, and later perfected by Robertson (22) for the determination of the individual blood proteins.

The clear supernatant fluid was drawn off from the tubes, after breaking them open near the top, and the refractive indices of each specimen determined in a Pulfrich refractometer. The readings are made in angles of total reflection and converted into indices of refraction by a table furnished with the instrument. The difference between the refractive index of the inactivated tube, serving as a control, and the tube containing the protein split-products represents the amount of egg white digested. To minimize the experimental error each series was done in duplicate using two control tubes and two active tubes, and as shown by the tabulated results, the duplicates agree to ± 0.00008 , which corresponds to an error of 1 second in reading.

Table I gives the complete results of this series of embryos and demonstrates a lack of a measurable amount of proteolytic

TABLE I.

Refractometric Determination of the Relative Amount of Protein Digested by Pancreatic Extracts from Pig Embryos of Various Ages, by Comparison of Differences of the Indices of Refraction in Active and Control Tubes (in Duplicate).

Size.	Approximate age. (After Coe, 24.)	Indices of refraction.		Refractivity due to the solution of split products. (Average of two determinations.)		
		(1)	(2)	Series 1.*	Series 2.†	Series 3.‡
mm.	days					
	Adult.	A- 1.34148			0.00088	
		C- 1.34059				
220	89	A- 1.33959		0.00072		
		C- 1.33887				
133	70	A- 1.34098			0.00074	
		C- 1.34024				
123	67	A- 1.34008	1.34008		0.00061	
		C- 1.33943	1.33951			
105	63	A- 1.33855	1.33855	0.00048		
		C- 1.33807	1.33807			
100	62	A- 1.34131	1.34131			0.00086
		C- 1.34049	1.34041			
95	61	A- 1.34016	1.34024		0.00065	
		C- 1.33959	1.33951			
92	60	A- 1.34065	1.34073			0.00094
		C- 1.33975	1.33975			
88	58	A- 1.34000	1.33992			0.00041
		C- 1.33959	1.33951			
78	55	A- 1.34163	1.34163		0.00016	
		C- 1.34147	1.34147			
78	55	A- 1.34081	1.34000			0.00024
		C- 1.34065	1.33967			
75	53	A- 1.34041	1.34041			0.00021
		C- 1.34016	1.34024			
72	52	A- 1.33727	1.33719	-0.00004		
		C- 1.33727	1.33727	0.00000		
65	50	A- 1.33975	1.33967			-0.00004
		C- 1.33975	1.33975			0.00000

A = Tubes containing active enzyme. C = Control tubes.

* Series started on May 25, 1918.

† Series started on May 30, 1918.

‡ Series started on June 4, 1918.

It is to be noted that the actual variance between the duplicate pairs (1), (2) always comes within the limit of the error of the apparatus (± 0.00008), for each determination. Results lying between 0 and ± 0.00008 have been taken as 0 since this is wholly within the magnitude of the experimental error.

type of zymogen in fetuses up to the age of 75 mm. From this age up to 92 mm. a rapid increase in the zymogen content is noted which then becomes practically constant for a unit quantity of gland. This increase in zymogen content is more clearly

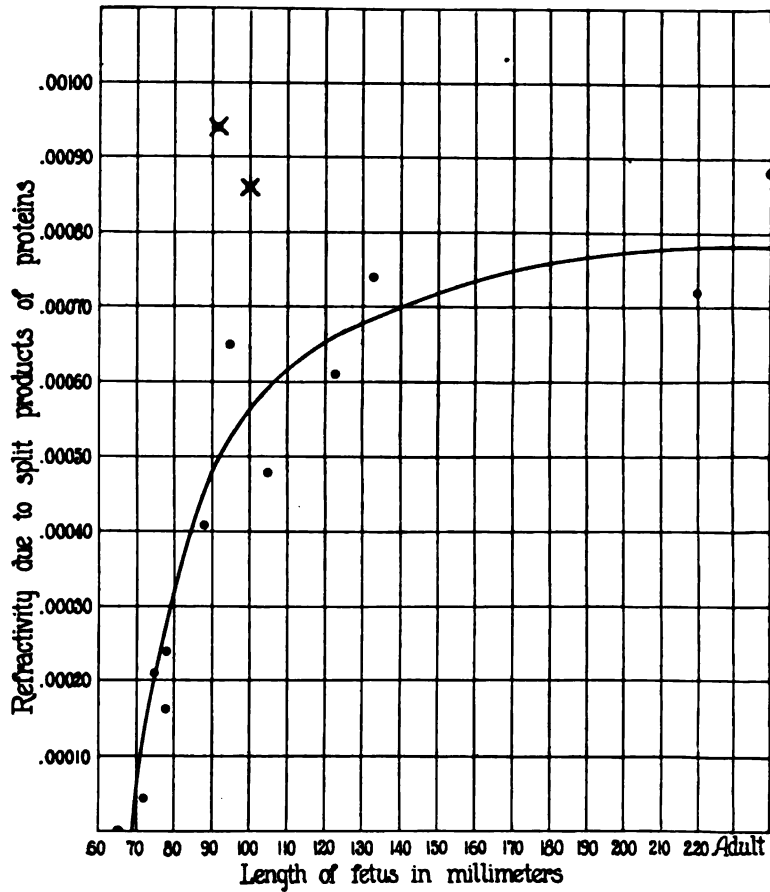


FIG. 1.

demonstrated in the curve approximating an average of the values plotted graphically (Fig. 1). The high digestive power of two tubes of the same series, which throws them out of the zone traversed by the curve, may be explained by certain factors

which would be constant for an individual series of a particular day. Since at best, an age-length ratio is only a rough estimate, the ages herein quoted as based on an unofficial statement of Coe (24), should be considered only as serving to give a general impression of the time prior to birth at which this alteration in zymogen content, as discussed above, occurs.

CONCLUSIONS.

1. Between the lengths of 65 and 72 mm. or the approximate intrauterine ages of 50 and 53 days of the pig embryos, the pancreas develops an amount of trypsinogen, which, when activated, is sufficient to digest enough protein to be measured by the refractometric method.

2. At a length of 95 mm. or an intrauterine age of from approximately 60 to 62 days, a maximum is reached in the trypsinogen content.

3. Although the two facts may not be related, the age of onset of a measurable amount of trypsinogen in the pancreas agrees closely with the time at which Corner has shown that a dendritic duct system is substituted for the primitive anastomotic tubules.

Since this study has shown that by the methods employed the accumulation of recognizable zymogen in the pancreas has a rather definite point of beginning and reaches a definite maximum, it becomes increasingly interesting to see whether finer structural details in the pancreas are coincident with this. It will be recalled that Bensley (25) and other workers claim to have recognized cytological criteria for enzyme-secreting cells. These morphological aspects of the question will constitute a later communication by Dr. Corner.

I am indebted to Dr. T. B. Robertson for his assistance in suggesting alterations toward perfecting the method of attack on this problem; to Dr. H. M. Evans for the original suggestion; and to Dr. C. L. A. Schmidt for his aid in revision of the manuscript.

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THE METABOLISM OF BILE ACIDS.

I. A QUANTITATIVE METHOD FOR ANALYSIS OF BILE ACIDS IN DOG'S BILE.

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An unusual opportunity for a comprehensive study of the bile acids presented itself to us in this laboratory because of the presence of a number of bile fistula dogs under *careful routine* observation. These bile fistula dogs were under observation for the study of bile pigments. They present normal factors of weight, activity, and appetite when under the laboratory routine care which has been described in detail by Hooper and Whipple (8).

In order to study satisfactorily the metabolism of the bile acids it is necessary to have a method for the analysis of bile acids which is relatively simple and accurate and which does not require large quantities of material for analysis. The method must be specific for bile acids and react negatively with the other substances in bile and it must not be obscured by any substances which may be present in bile fistula bile. We know of no published method which meets all these requirements.

As a preliminary step we undertook to test a number of the clinical methods for the determination of bile acids, some of which are known to be inaccurate and others of which are claimed to be merely qualitative. Without exception we have found these

*This series of papers on Bile Acid Metabolism was completed just prior to the death of Miss Foster from influenza pneumonia. The work should stand as a memorial to her enthusiasm, patience, and spirit of truthful research. This work was submitted as a thesis for her degree of Doctor of Philosophy, University of California.

relatively simple clinical methods to be grossly inaccurate and useless for analysis of bile salts. This statement applies to a recent method advocated by Hoover and Blankenhorn (9). No controls are given by these workers to show that many other substances present in abnormal sera may not have been responsible for the positive bile acid reaction noted in their experiments. For a discussion of these possibilities refer to the paragraph below on Pettenkofer's test.

Some of the more elaborate chemical methods for the extraction of bile salts from bile are relatively accurate, but the large quantity of material used and the amount of time required make frequent analyses at short intervals impracticable. These extraction methods are very expensive as well as time-consuming. The older methods may be divided into three groups: (1) methods in which the bile acids are separated and weighed; (2) methods in which the bile acids are calculated from the sulfur content in whole bile; and (3) methods which depend on the color reactions of cholic acid. There are possibilities of error in all these methods; as will be pointed out later.

Methods in Which the Bile Acids are Separated and Weighed.

Huppert's Method—1884.—Huppert (11) modified Neukomm's (17) method for bile salts in urine and applied it to the bile and blood in the following way. The albumin was first coagulated with alcohol and the precipitate carefully extracted with additional amounts of alcohol. The whole extract was evaporated, dissolved in water, freed from fat by ether extraction, neutralized, and $\text{Ba}(\text{NO}_3)_2$ added to separate the fatty acids, soaps, and any remaining protein. The washed precipitate was supposedly free from bile acids. The filtrate and wash water were precipitated with lead acetate and the precipitate washed with water to free from excess acetate. The precipitate was then washed out with alcohol, heated, and Na_2CO_3 added. After it was evaporated to dryness, the residue was extracted with absolute alcohol; the alcohol was evaporated to dryness; and the residue was dissolved in water. This water solution was filtered into a weighed dish and dried to constant weight.

Socoloff's Method—1875.—Socoloff (19) published a method in which the bile acids present were calculated by the amount of bile soluble in alcohol. This work was done in Hoppe-Seyler's laboratory using a method very much like the one described in the following paragraph.

Hoppe-Seyler's Method—1881.—Hoppe-Seyler's method (10) was used by Pfaff and Balch, Stadelmann, and others. The bile was dried to constant weight at 110°C . and the residue completely extracted with boiling

absolute alcohol. The extract was allowed to stand 24 hours, filtered, and evaporated to dryness at 110°C. The residue was dissolved in absolute alcohol, evaporated to a small volume, cooled, and precipitated with a large excess of absolute ether. The bile salts which crystallized out were dried at 110°C. and weighed. Stadelmann (21) evaporated the bile to a thick syrup and allowed it to stand in contact with alcohol on the water bath some time in order to coagulate the protein and give a clear filtrate. He then repeated the extraction three times with boiling 96 per cent alcohol. He dissolved the final precipitate in water and dried to constant weight.

Croftan's Method—1902.—Croftan (3, 4) estimated the bile acids by coagulating the albumin, evaporating the filtrate and washings, and precipitating with absolute alcohol to free from salts. The filtrate was diluted with water, precipitated with basic lead acetate and ammonia, and the precipitate extracted with absolute alcohol and filtered hot. This solution of bile salts was dried to constant weight. (According to Croftan the mucin holds with it a large amount of bile acid and they are so closely bound that it is impossible to separate them even by repeated washings.)

Goodman's Method—1906.—Goodman (5) hydrolyzed 50 gm. of bile with 125 gm. of 60 per cent KOH in a reflux condenser for 24 hours. The solution was extracted five times with 75 cc. of freshly distilled petroleum ether in order to extract the cholesterol. The last of the petroleum ether was evaporated off on a water bath and the solution precipitated with 5 per cent BaCl_2 to separate the higher fatty acids. The precipitate was extracted with boiling water and the filtrate and washings evaporated to 200 to 300 cc. The solution was cooled in ice and salt, and acidified with HCl. After standing 2 hours the cholic acid which crystallized out was filtered off, washed, dried, and extracted in a Soxhlet apparatus with acetone. In about 5 hours all the cholic acid was dissolved and the solution was evaporated and dried to constant weight.

Methods in Which the Bile Acids Are Calculated from the Sulfur Content in Whole Bile.

Spiro's Method—1880.—The sulfur method as used by Spiro (20) consisted in fusing a definite amount of bile (usually 50 cc.) in a silver dish with KOH and KNO_3 . The mixture was dissolved in water, supersaturated with HCl, and precipitated hot with BaCl_2 . This precipitate was filtered on a paper of known ash, washed well, and dried. The main part of the precipitate was transferred to a porcelain crucible. The paper with the remainder of the precipitate was ignited and weighed. The main part of the precipitate was weighed, a few drops of concentrated H_2SO_4 added, washed out well with water into another filter paper, and this was weighed. The difference in weight showed the amount of the impurity. From this difference was calculated the BaSO_4 in the other portion of the precipitate which was weighed with the ash of the larger filter paper, and the total amount of sulfur was calculated from this corrected value.

Von Bergman's Method—1904.—Von Bergman (2) precipitated a 24 hour collection of bile with many volumes of 96 per cent alcohol. The mucin precipitate was filtered off and washed six or eight times with alcohol. The combined washings and filtrate were evaporated to a definite volume and an aliquot part used for the determination of the combined sulfur.

Methods Based on the Color Reactions of Cholic Acid.

Pettenkofer's Method.—This test as outlined in Hammarsten (6) is performed by dissolving a small amount of bile in concentrated sulfuric acid and warming to 60 or 70°C. A 10 per cent solution of cane sugar is added drop by drop. A beautiful red color develops which turns bluish violet on standing. The red liquid shows a spectrum with two absorption bands, one at F and one between D and E. The test fails if the solution is heated too hot, or if too much sugar is added (on account of the sugar carbonizing). Also, if impurities are present in the acid, such as H_2SO_3 or the lower oxides of nitrogen, the reaction fails. Proteins, amyl alcohol, oleic acid, morphine, etc., give a similar color so that it is necessary to carry out the spectroscopic examination also. Ville and Derrien (23) state that vanillin and anisaldehyde give the same color, and cholesterol gives a similar color. This method has been improved upon by Mylius and von Udránszky, who advise the use of a 1 per cent solution of furfural in place of the sugar. Von Udránszky (22) emphasizes the fact that pure bile acids are necessary and suggests decolorizing the bile with charcoal and using an alcoholic solution of the residue. To each cc. of alcoholic solution, add one drop of furfural and 1 cc. of concentrated sulfuric acid, and warm gently. This will detect $\frac{1}{10}$ to $\frac{1}{2}$ mg. of cholic acid. According to Hammarsten, the protein and fat should also be removed by neutralizing the bile and adding alcohol to at least 85 volumes per cent pure alcohol. The solution is filtered and the protein extracted with fresh alcohol, and the alcoholic extract evaporated to dryness. This residue is extracted with absolute alcohol, filtered, and the extract evaporated to dryness. The residue is extracted with ether, dissolved in water, and the solution precipitated with basic lead acetate and NH_4OH . The precipitate is washed and dissolved in boiling alcohol, filtered, and made alkaline with a few drops of $NaOH$. This solution is evaporated to dryness, the residue extracted with absolute alcohol, filtered, and precipitated with ether. This solution may be used for Pettenkofer's test, but even this may contain phosphatides and they give the same color reaction as do the bile acids.

Inouye and Ito (12) reported that when vanillin and concentrated sulfuric acid are added to solutions of bile acids a red line is formed at the line of contact. If the fluids are then mixed, the solution changes to a red-brown and then violet. When this solution is diluted with glacial acetic acid, an adsorption band is shown at B. This reaction is sensitive with taurocholic acid in a dilution of 1:11,000 and with cholic acid 1:22,000.

Jolles (13) published a method in which the bile acid (2 to 3 cc. of 1 per cent solution) is mixed with rhamnose (1 to 2 drops of 5 per cent solution)

and boiled with 2 to 3 cc. of concentrated HCl. A red color is produced followed by a green fluorescence which is due to the formation of methyl furfuraldehyde. The reaction can be carried out by 0.005 to 0.0001 gm. of pure acid. It is not affected by urea, albumin, carbohydrates, hydrocarbons, or acids of the aliphatic or aromatic series, glycocoll, taurine, or cholesterol.

Authors' Method.

Dog's bile contains only taurocholic and taurocholic acids, and on hydrolysis these split into taurine and cholic and cholic acids. Taurine is amino ethyl sulfonic acid, $\text{CH}_2\text{NH}_2\text{CH}_2\text{HSO}_3$. It acts like an α -amino-acid, and gives off its NH_2 quantitatively in 3 minutes in the Van Slyke amino nitrogen apparatus. This method consists in hydrolyzing a definite amount of bile with NaOH, thus splitting the bile acid, and then determining the amount of NH_2 in the taurine. Neither taurocholic nor taurocholic acid gives off NH_2 before hydrolysis. In other animals both taurocholic and glycocholic acids are present, and it is therefore not certain that the method in its present form can be applied to the bile of animals other than the dog.

For the determination 5 cc. of bile cleared in the centrifuge and measured in a calibrated pipette are precipitated with 40 cc. of 95 per cent alcohol, and heated to the boiling point to insure a complete solution of the bile acid which might be held with the mucin precipitate. After cooling, the mixture is made up to 50 cc. in a cylinder with 95 per cent alcohol, and passed through a dry filter paper. Two specimens of 20 cc. each are evaporated to dryness. One is washed out with water and made up to 10 cc. in a calibrated flask. 2 cc. samples of this are used to determine the amount of NH_2 present before hydrolysis. The other is washed out quantitatively with 6 cc. of 8 per cent NaOH into a test-tube. The test-tube is loosely stoppered and placed in a boiling water bath for 5 hours. The contents are washed out into a 10 cc. calibrated flask and made up to volume with distilled water. 2 cc. samples of this are used to determine the amount of amino nitrogen due to the hydrolysis of the bile acids. The nitrogen is then figured on the basis of 1 cc. of bile for both specimens by multiplying the mg. of amino nitrogen found by 2.5 and subtracting the unhydrolyzed amino nitrogen from that due to hydrolysis. This figure is multiplied by the total volume of

bile for the 6 hour collection, which gives the total output of amino nitrogen for 6 hours. The bile acids are figured as taurocholic acid by multiplying by 36.72, the factor obtained by dividing the molecular weight of taurocholic acid by the atomic weight of nitrogen.

Quantitative Estimation of Taurine.

Table I shows that taurine gives up its NH_2 quantitatively with 3 minutes shaking in the Van Slyke amino nitrogen apparatus. The reaction is complete in that time even at as low a temperature as 13°C .

TABLE I.
Quantitative Estimation of Taurine.

Taurine.	Nitrogen gas.	Temperature.	Pressure.	Correction.	$\text{NH}_2\text{-N}$ found.	$\text{NH}_2\text{-N}$ theoretical.
mg.	cc.	$^\circ\text{C}$.	mm.	cc.	mg.	mg.
46.00	9.11 9.10	18	760	0.10	5.157	5.152
9.785	2.06 2.07	22	756	0.10	1.095	1.096
10.58	2.17 2.17	13	759	0.14	1.189	1.185

Analysis of Sodium Taurocholate.

To test out the method a specimen of sodium taurocholate was analyzed for N, S, and ash, and the NH_2 yielded after hydrolysis. Table II shows that the content of both N and S of the sodium taurocholate used was about 85 per cent of their theoretical values. The 13.3 per cent ash partly accounts for the low values of N and S. Sodium taurocholate should have had only 4.3 per cent sodium in the ash if one hydrogen atom was replaced by Na.

The solutions used in Table III were hydrolyzed 5 hours in a boiling water bath and made up to 10 cc. in a calibrated flask. NH_2 determinations gave the following results.

Number.	Amount used.	Nitrogen gas.	Temperature.	Pressure.	Correction.	Amino nitrogen in 10 cc. solution.	Total NH ₃ -N in 10 cc.	Hydrolysis $\frac{\text{NH}_3}{\text{N}}$.
	cc.	cc.	°C.	mm.	cc.	mg.	mg.	per cent
1	1	1.83 1.82	20	758	0.10	9.74	10.29	94.7
2	1	1.28 1.26	20	758	0.10	6.62	6.86	96.5
3	2	1.31 1.31	20	758	0.10	3.42	3.43	99.8
4	2	0.33 0.33	20	758	0.10	0.65	0.68	95.6

TABLE II.
Analysis of Sodium Taurocholate.

Sodium taurocholate.	Total N.		Theoretical N.	Percent of theoretical value.	Ash.
mg.	mg.	per cent	per cent	per cent	per cent
779.6	17.15	2.20	2.60	84.62	13.3

Sodium taurocholate.	BaSO ₄	Total S.	Theoretical S.	Per cent of theoretical value.
mg.	mg.	per cent	per cent	per cent
250*	92.6	5.09	5.96	85.42

*The sulfur determinations were very kindly carried out by Dr. C. L. A. Schmidt.

TABLE III.
Hydrolysis of Sodium Taurocholate.

Number.	Sodium taurocholate solution.*		Water added.	Sodium taurocholate.	16 per cent of NaOH added.	NaOH in solution.
	cc.	mg.	cc.	per cent	cc.	per cent
1	3	467.7	0	15	3	8
2	2	311.8	1	10	3	8
3	1	155.9	2	5	3	8
4	3†	31.2	0	1	3	8

* 3.8980 gm. were made up to volume in a 25 cc. flask and definite amounts were used.

† Solution prepared by diluting 1 cc. of the original solution with 14 cc. of distilled water.

Table III shows that the hydrolysis of this specimen of sodium taurocholate in from 1 to 15 per cent solutions was approximately 100 per cent in 5 hours when the alkali present was 8 per cent during the hydrolysis.

This complete analysis was carried out on another specimen of sodium taurocholate with the same results.

Normal Constituents of Whole Bile.

According to Hoppe-Seyler (10) the normal constituents of whole bile are bile salts, bile pigments, cholesterol, mucin, ethereal sulfates, conjugated glucuronic acids, fats, soaps, a trace of urea, jercorin and other phosphatides, hydrochloric and phosphoric acids, and sulfuric acid as Na, P, Ca, Mg, Fe, and Cu salts. Marshall and Davis (16) found the same amount of urea in the bile as in the blood—32 mg. of urea per 100 cc. 1 cc. of bile would then contain 0.149 mg. of N, but Van Slyke states that only 3 per cent of N in the urea is given off in the first 3 minutes, *i.e.* 0.00447 mg. of N, and that amount is not sufficient to cause an appreciable error in this method. Traces of amino-acids have been found in disease, but these would give off their NH_2 nitrogen in the unhydrolyzed specimen, and thus would be corrected for. Stadelmann thinks that glycocholic acid may be present in dog's bile. The generally accepted opinion is that dog's bile contains no glycocholic acid or at the most a small trace. Even the presence of glycocholic acid sufficient to make up 10 per cent of the hypothetical bile acid mixture, would introduce no appreciable error, figuring all the bile acids as taurocholic acid. This is because of the large size of the cholic acid molecule and the similarity of weight of the molecules of glyocol and taurine. None of the other constituents set free NH_2 groups on hydrolysis except mucin. To obviate this error we precipitated the mucin with 10 volumes of 95 per cent alcohol. In one set it was filtered immediately; in a second, the alcohol was allowed to remain in contact with the mucin for 24 hours; and in a third, the alcoholic mixture was heated to its boiling point, cooled, and filtered immediately. Since Croftan showed that the mucin precipitate could not be freed of bile acid by extraction, we made the mixture to a definite volume with alcohol and then took an aliquot part of the filtrate. This would insure a uniform mixture of bile salts.

Table IV shows that mucin is best removed by alcohol heated to its boiling point. The NH_2 given off by the unhydrolyzed bile may be due to traces of urea or to some splitting of the bile acid. It is always proportional to the bile acid content, but not in exact ratio. The unhydrolyzed mucin-free bile gives a higher NH_2 in some cases than whole bile. This may be due to a slight splitting of the bile acid. The hydrolyzed whole bile gives off decidedly more NH_2 nitrogen than the mucin-free bile. This shows that the mucin is decomposed by hydrolysis into NH_2 -

TABLE IV.
Whole Bile and Mucin-Free Bile.

Dog.	Unhydrolyzed bile.		Hydrolyzed bile.			
	Whole.	Mucin-free.	Whole.	Mucin-free.		
	$\text{NH}_2\text{-N}^*$ per cc. of bile.	$\text{NH}_2\text{-N}$ per cc. of bile.	$\text{NH}_2\text{-N}$ per cc. of bile.	$\text{NH}_2\text{-N}$ per cc. of bile filtered immediately.	$\text{NH}_2\text{-N}$ per cc. of bile after 24 hours.	$\text{NH}_2\text{-N}$ per cc. of bile heated to boiling.
	mg.	mg.	mg.	mg.	mg.	mg.
17-151	0.194	0.245	1.210	0.924	1.012	0.915
16- 15	0.051	0.072	0.277	0.188	0.144	0.203
15- 22	0.205	0.245	1.386	1.185	1.214	1.276
18- 23	0.080	0.072	0.452	0.216	0.289	0.290

* The readings on the NH_2 apparatus have been omitted for simplicity.

containing substances (amino-acids). The mucin precipitated by heating the mixture of bile and alcohol to boiling seems to give the quickest and most satisfactory results.

Sodium Taurocholate Added to Bile.

To ascertain if the amino nitrogen determined in hydrolyzed bile is really specific for the bile acid, three specimens of the same bile were hydrolyzed after adding different known amounts of sodium taurocholate to each. A control was run on the bile and on the sodium taurocholate solution.

Table V shows that known amounts of sodium taurocholate added to bile can be recovered quantitatively with this method. The theoretical increase is figured from No. 1. The error is about 6 to 8 per cent loss. Since our conclusions are based on

decided increases in bile acid excretion in our experiments, a loss due to the method would have little significance in the analysis of results.

TABLE V.
Known Amounts of Sodium Taurocholate Added to Bile.

Number.	Bile.	Sodium taurocholate. (15 per cent solution).	NH ₂ -N increase.	NH ₂ -N increase due to sodium taurocholate.	Theoretical increase.	Error (loss).
	cc.	cc.	mg.*	mg.	mg.	per cent
1	0	1		1.150		
2	5	3	4.061	3.229	3.45	6.4
3	5	2	2.953	2.121	2.30	7.8
4	5	1	1.914	1.082	1.15	6.1
5	5	0	0.832			

* The readings for the NH₂ have been omitted for simplicity.

A Six Hour Collection of Bile.

Table VI shows the same total, for three periods of 2 hours each as for one period of 6 hours. This speaks for a thorough mixture of the thick viscous bile and its contained bile acids and shows that a single estimation is accurate for the total excretion. It also gives confirmatory evidence of the accuracy of this method.

TABLE VI.
Mixed Collection of Bile.

Time.	NH ₂ -N per 1 cc. of bile.	Volume.	Total NH ₂ .	
			Output.	Per 6 hours.
hrs.	mg.	cc.	mg.	mg.
1-2	1.174	15	17.61	
3-4	0.553	14	7.74	
5-6	0.558	16	8.93	34.28 Total.
1-6	0.753	45	33.88	33.88 Total.

Variations in the Concentration of Alkali.

The amount and concentration of alkali used are of minor importance as can be seen in Table VII. The same specimen of bile was hydrolyzed with varying amounts and concentration of

NaOH and the results show that the bile acid was completely hydrolyzed in all the tubes. Table VII also shows that slight variations in the amount and concentration of alkali used for the hydrolysis do not affect the accuracy of the method.

TABLE VII.
Strength of NaOH Used.

Tube.	NaOH		NH ₃ -N
	cc.	per cent	mg.
1	5	8	0.364
2	6	8	0.364
3	7	8	0.350
4	6	6	0.350
5	6	12	0.364

DISCUSSION.

Hammarsten (6 and 7) found jecorin, lecithin, and other phosphatides in bile, and all contain nitrogen and sulfur. Since the nitrogen is in the choline radical, it does not interfere with the present method, as choline on hydrolysis with alkalies yields trimethylamine and glycol, and trimethylamine does not react with nitrous acid. But sulfur does interfere with the bile acid methods as determined by the sulfur content.

The phosphatides cause a decided error in the methods in which the bile acids are weighed. Long and Gephart (15) have found it impossible to separate bile acids from lecithin even with acetone. Bile salts can hold in stable solution 80 per cent of their weight of egg lecithin. Part of this can be separated by precipitation, but the amount remaining with the bile acids and not separated by acetone is much in excess of that contained in any bile. Ethereal sulfates are present in some bile (human and shark) according to Hammarsten, and thus interfere with the sulfur determination. But von Bergman could not detect any in dog's bile. It is evident that there are many sources of error in all the older methods.

The method outlined in this paper is not open to the criticisms of the methods previously used for the quantitative estimation of the bile salts. It is a simple procedure requiring careful technique only in washing out the various residues and making up to volume.

The results, although 6 to 8 per cent below the theoretical values, are very constant, as we have demonstrated over and over again when duplicates were run through by several different people. The determinations can be carried out so that the results are available within 8 hours.

SUMMARY.

A method is given for the quantitative estimation of the bile acid present in dog's bile. It is based on the fact that taurine gives up its NH_2 nitrogen quantitatively in the Van Slyke amino nitrogen apparatus. The taurocholic acid is hydrolyzed by NaOH into taurine and cholic acid. The amino nitrogen of the taurine is then determined by the gasometric method.

We are indebted to both Dr. Alice Rohde and Dr. Donald D. Van Slyke for their advice and help in working out this method; also to Dr. C. L. A. Schmidt for sulfur determinations.

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THE METABOLISM OF BILE ACIDS.

II. NORMAL FLUCTUATIONS IN HEALTHY BILE FISTULA DOGS.

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As a preliminary to any series of bile acid experiments it is necessary to establish the normal curve of excretion. This curve of excretion may vary with the condition of the animal, with the diet administered, and with other factors which may not be subject to control. Unless otherwise noted the dogs used in these experiments were in fine physical condition as shown by normal activity, vigorous appetite, a uniform weight curve, and normal blood picture. The operative procedure and general routine care of these animals have been described in detail in an earlier publication, Hooper and Whipple (1). It is very essential that these dogs be exercised, fed, set up, and drained regularly. Diet regulation is very important and at times difficult. Diarrhea is a troublesome feature which must be controlled, and this is often best done by a proper admixture of kaolin to the food. We have found it necessary to set up the dogs for collection of bile at least 30 minutes before the actual collection is started. This assures a complete drainage of the thick viscid night bile which escapes only slowly from the fistula. The presence of this concentrated bile in the first collection will cause high readings and introduce an error unless this precaution is taken. This explains some of the high readings in the first 2 hour periods of Tables VIII and IX. Unless otherwise stated, dogs were fed exactly 2 hours after the daily collection was started and again after the period of exercise following the collection. Weights were taken in the morning before the collection.

EXPERIMENTAL.

The method of chemical analysis has been controlled and shown to be reasonably accurate. The method of bile collection has been described and may be assumed to be accurately controlled. The dogs were kept in a room in which they were under constant supervision, which assured an accurate and complete bile collection. Occasionally a dog may be restless and displace the rubber tube in the fistula allowing the escape of bile, but the binders are large and by experience carefully fitted, so that such accidents are rare. When there was loss of bile the material was discarded unless the loss was very small or could be measured. Notes are made of any deviations from the uniform routine complete collection. As explained in another place we feel that 6 or 8 hour collections are more satisfactory than the longer 12 to 24 hour collections used by Stadelmann and other workers. The longer collections are more trying to the animal and usually cause loss of weight, appetite, and strength. This immediately introduces the factor of disease with its many unknown variables. We feel that these 6 hour collections continued over weeks and months in dogs which are in every respect healthy and active will give more truthful information about the normal bile acid metabolism.

It is to be noted in the first two tables (Tables VIII and IX) that the dogs were not drained for 30 minutes before the collections were begun. The first 2 hour periods show a bile which is more concentrated and contains more bile acids than does the second unit period. A part of this high excretion during the first 2 hours is undoubtedly to be explained by the presence of some of the concentrated night bile in the bile passages, not completely drained off before collections were begun. One must keep in mind the normal fluctuations in bile acid excretion on a mixed diet so that proper care may be exercised in the interpretation of the fluctuations noted under experimental conditions. Fluctuations in bile volume are at times startling and inexplicable. The constitution of the mixed diet may account for some of the fluctuations in bile volume. It has been pointed out elsewhere by Whipple and Hooper (4) that a meat diet produces a thin, pale, voluminous bile excretion, poor in bile pigments; and further

TABLE VIII.

*Bile Acid Excretion—2 Hour Periods—Mixed Diet.**Dog 18-23. Simple Bile Fistula.*

Date.	Hour.	Bile.	Amino nitrogen.		Taurocholic acid output.	Weight.	Remarks.
			Per cc. of bile.	Output.			
<i>1918</i>		<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>lbs.</i>	
Mar. 5	1-2	20	0.374	7.48			Mixed diet.
	3-4	26	0.231	6.00			
	5-6	29	0.158	4.58			
	1-6	75		18.06	664	32.0	
Mar. 6	1-2	17	0.344	5.85			
	3-4	26	0.228	5.93			
	5-6	21	0.198	4.16			
	1-6	64		15.94	585	32.0	
Mar. 7	1-2	25	0.253	6.32			Hb. 110 per cent. R. B. C. 6,290,000.
	3-4	28	0.170	4.76			
	5-6	32	0.114	3.65			
	1-6	85		14.73	541	33.3	
Mar. 8	1-2	22	0.264	5.80			
	3-4	34	0.190	6.46			
	5-6	25	0.191	4.77			
	1-6	81		17.03	626	34.0	
Mar. 11	1-2	34	0.247	8.40			
	3-4	32	0.160	5.11			
	5-6	25	0.189	4.72			
	1-6	91		18.23	670	32.8	
Mar. 12	1-2	23	0.253	5.82			
	3-4	27	0.148	3.99			
	5-6	19	0.153	2.90			
	1-6	69		12.71	467	34.0	
Mar. 13	1-2	28	0.368	10.30			
	3-4	29	0.368	10.67			
	5-6	25	0.225	5.63			
	1-6	82		26.60	978	35.0	
Mar. 14	1-2	21	0.315	6.61			
	3-4	21	0.286	6.60			
	5-6	18	0.300	5.40			
	1-6	60		18.61	662	35.5	
Mar. 15	1-2	34	0.390	13.26			
	3-4	22	0.277	6.09			
	5-6	26	0.223	5.80			
	1-6	82		25.15	923	34.8	

TABLE IX.

*Bile Acid Excretion—2 Hour Periods—Mixed Diet.**Dog 17-151. Simple Bile Fistula.*

Date.	Hour.	Bile.	Amino nitro- gen.		Tauro- cholic acid output.	Weight.	Remarks.
			Per cc. of bile.	Out- put.			
1918							
Mar. 5	1-2	7	0.828	5.79	1,196	42.5	
	3-4	26	0.675	17.55			
	5-6	28	0.329	9.21			
	1-6	61		32.55			
Mar. 6	1-2	11	1.26	13.86	1,167	41.8	
	3-4	23	0.549	12.62			
	5-6	14	0.379	5.30			
	1-6	48		31.78			
Mar. 7	1-2	24	0.409	9.81	852	42.5	Hb. 118 per cent. R. B. C. 6,350,000.
	3-4	25	0.267	6.67			
	5-6	31	0.213	6.70			
	1-6	80		23.18			
Mar. 8	1-2	13	0.618	8.03	818	43.0	
	3-4	6	0.816	4.89			
	5-6	13	0.717	9.32			
	1-6	32		22.24			
Mar. 11	1-2	13	0.437	5.68	400	41.5	
	3-4	15	0.278	4.17			
	5-6	10	0.101	1.01			
	1-6	38		10.86			
Mar. 12	1-2	13	0.780	10.14	850	43.0	
	3-4	13	0.780	10.14			
	5-6	4	0.717	2.86			
	1-6	30		23.14			
Mar. 13	1-2	10	1.00	10.00	809	42.5	
	3-4	3	0.691	2.07			
	5-6	15	0.663	9.94			
	1-6	28		22.01			
Mar. 14	1-2	32	0.656	20.99	906	43.5	2nd and 3rd collec- tions combined.
	3-4	8	0.457	3.65			
	5-6						
	1-6	40		24.64			
Mar. 15	1-2	13	0.717	9.32	559	44.0	2nd and 3rd collec- tions combined.
	3-4	5	0.654	5.88			
	5-6	4					
	1-6	22		15.20			

that a carbohydrate diet is associated with a thick, scanty bile excretion, rich in bile pigments. The mixed diet used in these experiments consists of a variable mixture of kitchen scraps containing meat, bones, bread, potato, rice, soup, etc.

Table VIII shows the variations of bile salt excretion from day to day in 2 hour collections. No preliminary drainage.

Table IX shows the variation in bile acid excretion from day to day in 2 hour collections. No preliminary drainage.

Hourly Variations in Bile Acid Elimination.

Dogs were set up for 8 and 9 hours to establish the hourly curve of daily excretion of bile acids. Compare Tables X and XI with Tables VIII and IX of this paper and subsequent tables in Paper III. These experiments are characteristic of many others which need not be tabulated at this time, but some of these supplementary observations will be given in later papers to prove other points.

TABLE X.

*Bile Acid Excretion—Hour Periods—Mixed Diet.
Dog 18-137. Simple Bile Fistula and Splenectomy.*

Hour.	Volume.	Amino nitrogen.		Taurocholic acid per hour.	Remarks.
		Per cc. of bile.	Per hour.		
	cc.	mg.	mg.	mg.	
1	9.0	0.317	2.85	104	October 1.
2 and 3	20.0	0.302	3.00	110*	
4	9.0	0.274	2.47	90	
5	12.0	0.230	2.76	101	End of 5th hour fed mixed diet.
6	10.5	0.230	2.41	88	
7 and 8	22.0	0.216	2.37	87*	Hb. 90 per cent. R. B. C. 3,830,000. Weight 24.0 lbs.
9	10.5	0.230	2.41	88	

* Average of 2 hours.

Table X is quite typical of a group of experiments and shows a nearly uniform hourly elimination of bile acids. There is a general tendency for the bile acid excretion curve to fall slightly in the afternoon in spite of a midday feeding and careful preliminary drainage of the concentrated night bile before beginning the experiment.

TABLE XI.

*Bile Acid Excretion—Hour Periods—Meat Diet.**Dog 17-34. Bile Fistula and Splenectomy.*

Hour.	Volume.	Amino nitrogen.		Taurocholic acid per hour.	Remarks.
		Per cc. of bile.	Per hour.		
	cc.	mg.	mg.	mg.	
1	3.8	0.686	2.61	96	December 11.
2	9.1	0.438	3.98	147	Fed 500 gm. of meat.
3	9.1	0.351	3.19	118	" 300 " " "
4	6.4	0.614	3.93	143	Weight 29.8 lbs.
5	4.1	0.614	2.52	92	
6	5.7	0.614	3.50	129	
7	5.1	0.658	3.36	124	
8	3.9	0.731	2.85	106	
9	6.0	0.686	4.10	150	

TABLE XI-a.

*Bile Acid Excretion—2 Hour Periods—Fasting and Meat Diet.**Dog 17-34. Bile Fistula and Splenectomy.*

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 2 hours.	Bile pigments in 2 hours.	Remarks.
		Per cc. of bile.	In 2 hours.			
	cc.	mg.	mg.	mg.	mg.	
1-2	22	0.440	9.68	355	15.3	February 13.
3-4	20	0.332	6.64	242	13.2	Fasting. Mixed diet day before fasting.
5-6	20	0.318	6.36	235	13.9	Weight 31.8 lbs.
7-8	18	0.303	5.45	201	13.1	
9-10	19	0.318	6.04	220	7.0	

February 19 to 26—Rice, potato, and milk diet.

1-2	21	0.189	3.96	146	11.0	February 27.
3-4	16	0.132	2.11	77	8.9	Meat 250 gm. at beginning.
5-6	20.5	0.161	3.30	121	12.4	Weight 27.5 lbs.
7-8	14	0.247	3.46	129	11.2	
9-10	13	0.261	3.39	124	11.8	

Tables XI and XI-a show slight fluctuations in bile acid output after feeding, but we do not attach any significance to this reaction. It will be noted (Table XI-a) that the level of bile

TABLE XII.

*Bile Acid Excretion Not Influenced by "Bile Exclusion."**
Dog 18-23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
June 3	62	0.326	20.20	741	6.5	30.2	No bile exclusion.
" 4	60	0.409	24.54	902	13.6	31.2	Mixed diet.
" 5	61	0.422	25.74	932	20.9	31.2	
" 6	82	0.371	30.42	1,115	11.2		
" 7	75	0.191	14.33	526	24.8	32.2	
" 8	58	0.284	16.57	608	16.2	32.0	Hb. 127 per cent. R. B. C. 6,665,000.
" 10	75	0.224	16.80	618	22.2	30.2	
" 11	77	0.289	22.25	817	28.7	31.7	
Average.....			21.4	782	No bile exclusion.		
June 12	80	0.199	15.92	584	23.7	32.0	Absolute bile exclusion.
" 13	78	Lost.			16.3	32.0	Mixed diet.
" 14	96	0.346	33.22	1,215	20.0	32.0	
" 15	72	0.248	17.86	656	30.2		
" 16							Dog set up and drained 2 hrs.
" 17	66	0.180	11.88	436	24.1	30.5	
" 18	83	0.238	19.75	725	23.3	32.0	
Average.....			19.72	723	Complete bile exclusion.		

* "Bile exclusion" means total inability of the dog to lick any bile from the fistula at any time. This is effected by means of a thick gauze pad and large binder.

acid excretion is influenced by the diet of the previous day. This point will be taken up again.

This method makes it possible to follow the hourly fluctuations in the bile acid output and to establish for the first time the actual hourly curve of bile acid elimination from hour to hour.

TABLE XIII.

*Bile Acid Excretion Not Influenced by "Bile Exclusion."
Dog 18-23. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
May 21	57	0.223	12.72	467	12.9	30.75	No bile exclusion. Diet 300 gm. of cracker meal, 65 gm. of meat.
" 22	52	0.255	13.26	487	10.8	30.75	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 23	73	0.323	23.58	866	12.9	30.50	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 24	61	0.253	15.44	567	12.9	30.25	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 25-26						30.25	Mixed diet.
" 27	66					30.25	300 gm. of cracker meal, 65 gm. of meat.
" 28	72	0.221	15.90	574	7.5	30.50	300 gm. of cracker meal, 65 gm. of meat.
" 29	82	0.334	27.40	1,005	11.2	30.25	300 gm. of cracker meal, 65 gm. of meat.
" 30							300 gm. of cracker meal, 65 gm. of meat.
" 31	72	0.210	15.12	552	10.0	30.00	
Average.....				17.6	645		No bile exclusion.
June 19	77	0.252	19.40	712	18.8	32.50	Absolute bile exclusion. 300 gm. of cracker meal, 65 gm. of meat.
" 20	78	0.210	16.38	602	23.7	32.00	300 gm. of cracker meal, 65 gm. of meat.
" 21	59	0.294	17.35	637	19.8	31.50	300 gm. of cracker meal, 65 gm. of meat.
" 22	28	0.476	13.32	489	23.4	31.00	300 gm. of cracker meal, 65 gm. of meat.
Average.....				16.6	610		Complete bile exclusion.

The question of "bile exclusion" is a very important one for this entire series of experiments. The question resolves itself into the following: Does a bile fistula dog lick enough bile from

its fistula during the night to influence in any way the daily excretion of bile or bile acids? According to Stadelmann (3) in his experiments this "bile exclusion" is necessary. The exclusion of any possible ingestion of the dog's own bile in his experiments would cause a decrease of about $\frac{1}{3}$ the total bile acid excretion. Because of our respect for Stadelmann's work we felt that it was necessary to control this point beyond the peradventure of a doubt. We submit a sufficient number of experiments in Paper V to prove that under the conditions of our experiments "bile exclusion" does not influence the output of bile acids. The reasons for this are discussed more in detail in that paper. These experiments (Tables XII and XIII) make the same point and are sufficient to inform the reader that this important factor has been properly controlled in experiments that follow. Absolute "bile exclusion" does not affect the output of bile acids on either a mixed diet or a known diet. The bile salt excretion can be partly controlled by diet, as will be shown in another paper. A diet rich in meat protein increases the output and a diet poor in meat protein reduces the excretion.

Effect of Bile by Mouth on the Following Day's Excretion.

In order to find out if bile by mouth in moderate amounts affected the following day's excretion several dogs were given bile at night and the bile acid excretion followed.

Table XIV shows that bile feeding in the late afternoon does not influence the following day's excretion of bile acids. Many other experiments have been performed with identical results. It is apparent from experiments tabulated in Paper III that the greater part (about 80 per cent) of the bile acids ingested as bile will appear in the bile fistula bile within 4 hours. We may assume that only bile ingested during the early morning hours (5 to 8 a. m.) influences the bile collections in our experiments. There is no clinical evidence that the dogs lick any bile from their fistulas during this period and the experimental data confirm this point.

TABLE XIV.

*Bile Feeding at Night—Mixed Diet.
Simple Bile Fistula with Splenectomy.*

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
17-181	Average previous 10 days.			10.60	389		
	1918	cc.	mg.	mg.	mg.	lbs.	
	July 29						4 p.m. given 58 cc. of bile by stomach tube.
	" 30	36	0.267	9.61	352	25.50	5 p.m. given 60 cc. of bile by stomach tube.
	" 31	25	0.418	10.45	384	26.25	5 p.m. given 60 cc. of bile by stomach tube.
	Aug. 1	38	0.285	10.82	397	25.00	No bile given.
	" 2	37	0.244	9.03	332	25.25	Hb. 100 per cent. R. B. C. 4,500,000.
18-137	Average previous 10 days.			14.9	547		
	July 29					22.75	4 p.m. given 58 cc. of bile by stomach tube.
	" 30	50	0.310	15.50	569	23.50	4 p.m. given 60 cc. of bile by stomach tube.
	" 31	42	0.401	16.80	617	24.0	5 p.m. given 60 cc. of bile by stomach tube.
	Aug. 1	43	0.395	16.55	608	23.5	No bile given.
	" 2	30	0.145	4.35	159	22.75	Hb. 100 per cent. R. B. C. 4,425,000.

DISCUSSION.

Loeb (2) stated that the sulfur and nitrogen excretion in the bile is higher during the first 4 hours than during the second 4 hours after eating. From the tables given in this paper it is clear that the bile acid excretion is greater during the early part of the day than later in the afternoon. There is always a tendency to fall off during the 5th and 6th hours of the collection in spite of the ingestion of food. But in an individual the amount

excreted throughout the day is fairly uniform provided a fore-period of 30 minutes or longer for drainage of the bile fistula has been a part of the routine preceding the usual 6 hour collection.

It is evident that the amount of bile which these dogs normally lick from their fistulas during the hours they spend in their cages is insufficient to cause any variation in the following day's output (Tables XII and XIII). The only time our dogs are prone to lick their fistulas is just after the completion of the daily collection. But Table XIV shows that moderate amounts of bile at this time are excreted before the following day's collection is made, and do not affect the determination in any way.

SUMMARY.

Bile acid excretion in a healthy bile fistula dog given a mixed diet will show great variations from day to day.

The amount of bile acid excreted is usually somewhat higher in the morning than in the afternoon. This holds good even after complete drainage ($\frac{1}{2}$ hour) of the concentrated night bile, and in spite of liberal feeding 2 hours after collections are begun.

The amount of bile acid excreted hour by hour during any given day is fairly uniform.

The amount of bile which a dog may lick from its fistula during the afternoon and night resting period is not sufficient in our experiments to cause any demonstrable variation in the following day's excretion.

Moderate amounts of bile given by stomach in the late afternoon do not influence the following day's excretion of bile acids.

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THE METABOLISM OF BILE ACIDS.

III. ADMINISTRATION BY STOMACH OF BILE, BILE ACIDS, TAURINE, AND CHOLIC ACID TO SHOW THE INFLUENCE UPON BILE ACID ELIMINATION.

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The cholagogue action of whole bile given by stomach is a well known fact. It has been established by many experiments that the curve of bile volume excretion does not necessarily parallel the curve of excretion of bile pigments (Whipple and Hooper, 9). In other words it is possible to stimulate a free flow of bile which is poor in bile pigments, so much so that the total bile pigment output may fall to half normal during a period of active cholagogue excretion with twice normal output of bile. Hooper (3) has pointed out several factors which may contribute to this reaction. It is well to recall that taurocholic acid given by mouth has a marked cholagogue action—experiments given below add more data to establish this statement. An ether extract of dried bile, which of course contains a mixture of substances, has no cholagogue action, but inhibits the excretion of bile pigments. It is of considerable interest to know that certain substances can stimulate or inhibit the total bile excretion while influencing the output of the various bile constituents in the same or opposite direction.

In 1875 Socoloff (7) injected glycocholic acid into a dog and found an increased excretion but no increase in the per cent of bile acids. But his method was questionable and he used only one dog. Rutherford and Vignal (5) injected bile acids into the jejunum and found an increased output of bile acids, but they do not mention how the bile acids were estimated. To Schiff (6) is given the credit for having established the fact that there is a reabsorption of bile acid and this is often termed the "circulation

of the bile." This term "circulation of the bile" is used loosely by some writers and is at times misquoted to indicate a circulation of other of the substances found in the bile, for example, bile pigments. We have definite proof that bile pigments are not absorbed from the intestine (Hooper and Whipple, 4). At present we have little if any accurate knowledge about the many other substances present in fresh bile—they may be absorbed or not.

In giving dog's bile by mouth, Stadelmann (8) found that with doses of 2.0 or 2.5 gm. of bile salt, the cholagogue action lasted up to 24 or 36 hours, but the bile salt was excreted within 24 hours, usually within 10 hours. With 1.5 gm. of pure sodium glycocholate the whole amount was excreted within 12 hours. The greatest cholagogue action was during the first 6 hours. From then on it began to abate and was over within the next 12 hours. The bile salts may have been excreted in less than 10 to 12 hours, for Stadelmann performed bile salt analyses only upon these large collections.

With doses of 3 to 5 gm. morning and evening the salts and volume were tremendously increased but the excretion was not directly proportional to the amounts given. 5 gm. twice a day for 3 successive days kept both the volume and salts above normal for more than 8 days, even though they were continually decreasing. These experiments of Stadelmann's are in harmony with those tabulated below, and supplement our experimental data.

One is not surprised to observe in the experiments given below that the curves of whole bile excretion and bile acid excretion may run parallel after the oral administration of fresh dog's bile—moreover, on the contrary, that these curves may be widely dissociated. A moderate dose of whole dog's bile given by stomach will cause a distinct cholagogue action and a parallel increase in bile acids. With the fall in bile volume after 3 to 5 hours there is a fall in the bile acid output. It is possible to give sugar with concentrated bile by stomach tube and completely inhibit the cholagogue action while a great rise in output of bile acids is taking place. We believe this procedure gives a maximum concentration of bile acids in dog's bile, as high even as 7 to 9 per cent by weight. Perhaps this represents the maximum power of the liver cells to concentrate bile acids in whole bile, at least under the conditions of the experiment.

EXPERIMENTAL.

The first four tables (Tables XV to XVIII) are to be taken as a unit because they illustrate the uniformity of reaction which follows the ingestion of moderate amounts of fresh dog's bile. The taurocholic acid content varies from 0.63 to 1.83 gm. in any given single dose. The hourly curve of bile acid excretion is remarkably

TABLE XV.
Bile Feeding.
Dog 17-181. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	8.0	0.319	2.55	93	August 27. End of 1st hour given 50 cc. of whole dog's bile containing 752 mg. of taurocholic acid.
2	18.5	0.667	12.30	452	
3	11.0	0.662	7.28	267	
4	7.0	0.596	4.17	153	
5	6.5	0.333	2.16	79	End of 5th hour fed mixed diet.
6	7.0	0.222	1.55	56	
7	10.5	0.222	2.33	85	
8	10.0	0.210	2.10	77	
9	9.0	0.207	1.86	68	Hb. 100 per cent. R. B. C. 4,810,000.
10	10.0	0.221	2.21	81	Weight 25.3 lbs.

constant and usually shows that the largest elimination takes place during the first 3 hours after ingestion. Some experiments show the maximum elimination of bile acids during the first hour, again during the second or third hour. The bile acid concentration per cc. of the bile eliminated usually closely parallels the curve of total excretion. The cholagogue action is marked with the larger doses but is practically absent following the smallest dose.

Table XV shows that about 90 per cent. of bile acid in whole bile given by mouth is excreted within 3 hours.

TABLE XVI.

*Bile Feeding.**Dog 18-137. Bile Fistula and Splenectomy.*

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	10.0	0.442	4.42	162	September 5.
2	7.5	0.313	2.35	86	End of 2nd hour 75 cc. of bile containing 628 mg. of taurocholic acid.
3	11.0	0.470	5.17	189	
4	9.0	0.666	6.00	220	
5	8.5	0.696	5.92	217	End of 5th hour fed mixed diet.
6	7.0	0.464	3.25	119	
7	10.0	0.275	2.75	101	
8	8.0	0.261	2.25	82	Hb. 120 per cent. R. B. C. 5,060,000.
9	8.0	0.248	1.98	72	Weight 22.25 lbs.
10	7.0	0.221	1.55	56	

TABLE XVII.

*Bile Feeding.**Dog 18-23. Simple Bile Fistula.*

Hour.	Volume.	Amino nitrogen.		Taurocholic acid.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	7.5	0.364	2.73	100	August 6. End 1st hour given 100 cc. of bile containing 1.825 gm. of taurocholic acid.
2	22.0	0.757	16.65	612	
3	17.0	0.980	16.65	612	
4	20.5	0.925	18.95	696	
5	13.5	0.582	7.86	288	End of 5th hour fed mixed diet.
6	11.0	0.373	4.10	150	
7	10.5	0.276	2.90	106	Hb. 120 per cent. R. B. C. 6,375,000.
8	9.0	0.345	3.10	114	Weight 31.5 lbs.

TABLE XVIII.
Bile Feeding.
Dog 18-137. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	8.5	0.530	4.51	165	September 24.
2	11.0	0.430	4.73	173	End of 2nd hour 100 cc. of bile containing 951 mg. of taurocholic acid.
3	18.0	0.788	14.18	520	
4	16.0	0.845	13.51	499	
5	10.0	0.516	5.16	189	End of 5th hour fed mixed diet.
6	10.5	0.508	5.33	195	
7	11.0	0.328	3.58	131	
8	11.0	0.312	3.43	126	Hb. 90 per cent. R. B. C. 3,830,000.
9	10.0	0.260	2.60	95	Weight 23.3 lbs.

TABLE XIX.
Concentrated Bile Feeding.
Dog 17-34. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	7.5	0.546	4.09	150	October 15.
2	6.4	0.445	2.85	104	End 2nd hour 170 cc. of concentrated bile solution* containing 11.50 gm. of taurocholic acid given by stomach tube.
3	21.0	0.968	20.31	745	
4	26.0	1.430	37.20	1,365	Vomited about 200 cc. of fluid.
5	29.0	1.360	39.44	1,445	End 5th hour fed mixed diet.
6	34.3	1.220	41.88	1,535	
7	54.0	1.000	54.00	1,980	
8	18.0	0.657	11.82	433	Hb. 108 per cent. R. B. C. 5,700,000.
9	12.0	0.299	3.59	132	Weight 31.5 lbs.
Oct. 16	60.	0.313	18.80	690	Usual 6 hour collection.
Oct. 17	64.	0.328	21.00	752	Usual 6 hour collection.
Oct. 18	56.	0.300	16.80	616	Usual 6 hour collection.

* Solution prepared by evaporating bile to dryness, extracting with water, and centrifuging. The clear supernatant fluid is this bile solution.

Table XVI shows that about 60 per cent of bile acid administered by mouth was excreted within 4 hours. Note little if any cholagogue action.

Table XVII shows that about 90 per cent of bile acid administered by mouth was excreted in 4 hours.

Table XVIII shows that about 85 per cent of bile acid given in bile by mouth is excreted within 4 hours.

TABLE XX.
Concentrated Bile Feeding.
Dog 18-137. Simple Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
1	7.5	0.187	1.40	51	September 18.
2	12.0	0.330	3.96	145	End 2nd hour 370 cc. of bile solution† containing 11.60 gm. of taurocholic acid given by stomach tube.
3	16.5	0.729	12.00	440	Vomited 60 cc.
4	20.0	1.111	22.22	816	
5	16.0	1.200	19.20	704	End 5th hour fed mixed diet.
6	14.0	1.010	14.15	519	Hb. 120 per cent.
7, 8, and 9	45.0	0.750	11.24*	413*	R. B. C. 5,060,000. • Weight 23.5 lbs.

* Average of 3 hours.

† Solution prepared by evaporating bile to dryness, extracting with water, and centrifuging. The clear supernatant fluid is this bile solution.

Tables XIX and XX show remarkably well the intense reaction which may follow large doses of taurocholic acid by mouth. Whole bile was not given, but a crude watery extract of dried dog's bile which is rich in taurocholic acid. Vomiting occurred and the amount regurgitated is not known.

The cholagogue action is noted immediately and is sustained many hours and even days (Table XIX). The concentration of bile acids per cc. of bile is much above normal and the actual output of almost 2 gm. per hour is reached in one experiment. Large

amounts of bile acids seem to cause no ill effects, immediate or delayed, except some nausea and vomiting. What effect if any is produced in the general body metabolism is unknown.

Table XIX shows that bile acid fed by mouth in high concentration causes a great increase in bile acid output for 6 hours, and is a

TABLE XXI.
Concentrated Bile Feeding Plus Sugar.
Dog 15-22. Simple Bile Fistula.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	mg.	mg.	mg.	
					September 26.
1	21.5	0.675	14.50	532	
2	22.0	0.380	8.36	301	End of 2nd hour 125 cc. of bile solution* containing 8.770 gm. of taurocholic acid—100 gm. of sugar- water. Little vomiting.
3	15.0	1.54	23.10	848	
4	9.5	1.93	18.35	673	
5	14.5	2.31	33.50	1,230	End of 5th hour fed mixed diet.
6	15.5	2.35	36.45	1,340	
7	15.0	2.26	33.90	1,245	
8	11.0	1.80	19.80	727	Hb. 110 per cent. R. B. C. 5,280,000.
9	8.5	1.77	15.05	552	Weight 32.8 lbs.
Sept. 27.	37.0	1.19	44.00	1,615	Usual 6 hour collection.
Sept. 30.	12.0	1.18	14.10	518	Usual 6 hour collection.

* Bile evaporated to dryness, the residue extracted with water, and centrifuged. The clear supernatant fluid is this bile solution.

cholagogue for a much longer period. About 65 per cent was excreted in 6 hours.

Table XX shows that concentrated bile acid by mouth may cause a long delayed bile acid excretion.

Table XXI shows that bile acid plus sugar by mouth causes a delayed bile acid excretion with fairly low volume of bile. The extreme cholagogue action of bile acids may be completely

inhibited by simultaneous administration of a sugar solution by stomach. Sugar solutions alone will cause the excretion of a very concentrated bile but will not modify the bile acid curve. This

TABLE XXII.

*The Effect of Taurine and Cholic Acid Separately and Combined.
Dog 17-151.* Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Urinary N.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	lbs.	gm.	
Average 11 days.	15		8.0*	294			3.3	Hb. 138 per cent. R. B. C. 7,670,000.
Mar. 30.....	34	0.277	9.42	346	26.6	36.75	2.52	Before collection: 4 gm. of cholic acid in capsule.
" 31.....	29	0.133	3.86	142	19.4	36.60	3.25	
Apr. 1.....	18	0.312	5.61	206	23.5	36.50	2.41	Before collection: 0.75 gm. of taurine in 30 cc. of salt solution intravenously.
" 2.....	28	0.353	9.89	366	17.5	36.25	2.63	
" 3.....	45	0.797	35.85	1,316	17.3	35.80	2.63	Before collection: 3 gm. of mixture $\frac{1}{2}$ cholic acid, $\frac{1}{2}$ taurine in capsule.
" 4.....	19	0.467	8.88	326	18.2	35.75	2.30	
" 5.....	47	1.141	53.60	1,967	20.9	35.50	2.58	Before collection: 4 gm. of taurocholic acid in capsule.
" 6.....	15	0.402	6.03	220		35.00	2.52	Hb. 125 per cent. R.B.C. 6,980,000.

* This dog was kept on a diet of 75 gm. of cane sugar and 100 gm. of glucose during this entire experiment, as well as during the fore-period of 11 days.

experiment gives the highest concentration of bile acids per cc. of fistula bile (86 mg. per cc.). The sugar seems responsible for a definite delay in output of bile acids; but the output is enormous in spite of the low volume and slight delay in elimination.

Tables XXII and XXIII are companion experiments and show great uniformity of reaction. From these two and many other experiments it is established that taurine by mouth or intraven-

TABLE XXIII.

*The Effect of Taurine and Cholic Acid Separately and Combined.
Dog 18-23.* Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Urinary N.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	lbs.	gm.	
Average 12 days.	24		8.7*	319			3.10	Hb. 120 per cent. R. B. C. 7,895,000.
Mar. 30.....	45	0.551	24.75	909	18.0	27.7	3.27	Before collection: 3 gm. of cholic acid in capsule.
" 31.....	34	0.183	6.22	228	24.6	27.4	3.25	
Apr. 1.....	40	0.142	5.68	208	18.8	27.1	2.57	Before collection: 0.75 gm. of taurine in 25 cc. of salt solution intravenously.
" 2.....	30	0.240	7.20	264	14.6	27.0	2.74	
" 3.....	55	0.698	38.4	1,300	10.5	26.6	2.55	Before collection: 2.6 gm. of mixture $\frac{1}{4}$ cholic acid, $\frac{1}{4}$ taurine in capsule.
" 4.....	29	0.354	10.25	376	11.1	26.5	2.69	
" 5.....	56	0.867	48.53	1,780	22.4	26.4	2.58	Before collection: 3 gm. of taurocholic acid in capsule.
" 6.....	37	0.171	6.33	232	14.8	25.8	3.30	Hb. 120 per cent. R. B. C. 7,300,000.

* This dog was kept on a diet of 75 gm. of cane sugar and 75 gm. of glucose during this entire experiment, as well as during the fore-period of 12 days.

ously does not in any way influence the output of bile acids. This is true during fasting periods as well as for full diets.

Taurocholic acid by mouth is known to be a cholagogue and this

action is well shown in our experiments. About 40 to 50 per cent of the taurocholic acid is excreted in the bile during the 6 hour collection period. There may have been slight delay in excretion owing to the use of capsules rather than solutions.

Taurine plus cholic acid fed by mouth exerts the same influence on the bile fistula dog as does the pure taurocholic acid. In other words this synthesis can take place in the body with great ease and rapidity. The cholagogue action and increased bile acid output are identical, whether the mixture of taurine plus cholic acid is given or the pure taurocholic acid.

Cholic acid alone has less than usual influence upon the bile acid output if given after a long fasting period. There may be a little increase in bile acid excretion or there may be a decided increase. The reaction seems to depend upon the amount of taurine present in the body which is available to combine with the cholic acid radical. Note additional data in Table XXIV.

Table XXIV furnishes more interesting data concerning the feeding of cholic acid and its influence upon the bile acid excretion. A small dose (2.0 gm.) of cholic acid acts as a cholagogue but does not increase the output of bile acid after an 11 day fasting period. A larger dose (4.0 gm.) of cholic acid after 9 days fasting produces a cholagogue action and a definite increase in the taurocholic acid of the bile—an increase of about 100 per cent. The next day the same dose repeated gives the same cholagogue action, but little if any increase in the bile acid output. We may assume that the first dose of cholic acid combined with all the available taurine in the body and formed taurocholic acid to be eliminated in the bile. The second large dose given the next day found no taurine to complement the cholic acid and no taurocholic acid resulted. We see therefore that either taurine or cholic acid can act as limiting factors in the over-production of taurocholic acid. This is particularly true of cholic acid, which is probably the normal determining factor. Cholic acid given by mouth during periods of full diet is usually associated with a strong cholagogue action and a great increase in output of bile acids (Table XXIV—Dog 17-34). This indicates an abundant exogenous source of taurine in the common mixed diet.

Table XXV shows the curve which results from the feeding of sodium taurocholate. Its reaction seems to be identical with that

TABLE XXIV.
Cholic Acid Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
15-22	1918 Average.	cc. 16	mg.	mg. 10.0	mg. 367	lbs.	Average 3 days after 11 days fasting.
	May 4	35	0.231	8.09	297	28.0	2.0 gm. of cholic acid in capsule at beginning.
	June 3	16	0.510	8.16	299	29.5	After 8 days fasting.
	" 4	38	0.535	20.33	746	29.0	4.0 gm. of cholic acid in emulsion at beginning.
	" 5	35	0.250	8.75	321	28.4	4.0 gm. of cholic acid in emulsion at beginning.
17-34	Oct. 9	24	0.537	12.90	473	31.5	Mixed diet.
	" 10	114	0.579	66.00	2,420	31.5	4.0 gm. of cholic acid in 15 cc. of alcohol at beginning.
	" 11	128	0.478	61.2	2,250	31.5	4.0 gm. of cholic acid in 15 cc. of alcohol at beginning.

TABLE XXV.
Bile Salt Feeding.
Dog 15-22. Simple Bile Fistula.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 2 hours.	Remarks.
		In 1 cc. of bile.	In 2 hours.			
	cc.	mg.	mg.	mg.	mg.	
1-2	18	0.800	14.14	529	6.8	September 4, 1917. End 2nd hour 10 gm. of crude sodium taurocholate given by stomach.
3-4	58	1.711	99.26	3,640	18.4	
5-6	45	1.597	71.85	2,635	11.7	
7-8	22	0.642	14.12	518	8.4	Weight 35.0 lbs.

observed after taurocholic acid feeding. The cholagogue effect is pronounced and about 50 per cent of the taurocholate is recovered in the first 4 hours of collection. The crude taurocholate was not carefully prepared or analyzed, so that the actual percentage output cannot be estimated. The reaction subsides rapidly and falls almost to normal within 6 hours.

DISCUSSION.

Von Bergman (1) fed sodium cholate to dogs and found a decided increase in the sulfur content when only 1.0 gm. was given. But his dog weighed only 4.5 kilograms. A dose of 2.0 gm. caused an even greater increase, but did not double it. He also fed a dog of 8.5 kilograms sodium cholate (2.0 gm.) every day for 3 days. The first day there was a sufficient amount of sulfur excreted to account for all the cholate being changed into taurocholic acid. But each successive day the sulfur excreted was less. The third day it was not back to normal although it was much lower. He explains this by saying that the body could not furnish sufficient taurine to unite with the abnormally high amount of sodium cholate given. Our experiments show that a minimal amount of taurine is available after a long fasting period (Table XXIV). Our mixed diet was probably richer in sulfur than von Bergman's diet of 200 gm. of meat, 150 gm. of rice, and 30 gm. of casein. Note the high output with cholic acid feeding and a liberal mixed diet (Table XXIV).

Goodman (2) gave 0.6 gm. of cholic acid to a dog on one day and found a decided increase in the amount of bile and the cholic acid excreted. This experiment was done once on a dog of 4.5 kilograms weight on a diet of dog biscuit.

The *cholic acid* appears to be the important determining factor in the output of taurocholic acid, and it is of some importance to learn the source of cholic acid in the body, its true metabolic history, and its usefulness and ultimate fate in the body. The solution of these and other questions relating to cholic acid is not easy but will repay further investigation.

Feeding cholic acid causes a minimal reaction after long periods of fasting. Repeated doses of cholic acid during fasting result in complete failure of subsequent doses to call out an increase of bile acid excretion. This may be interpreted to mean that under

these conditions of fasting and repeated ingestion of cholic acid the available taurine is reduced close to zero. The cholic acid is unable to combine with taurine in the usual way and is eliminated in some other form, perhaps in the bile or elsewhere.

Feeding cholic acid without taurine in a liberal mixed diet will give a maximal cholagogue action and output of bile acids. Presumably under these conditions there is ample taurine to combine with the cholic acid. The result is a large increase in taurocholic acid in the bile. Probably under normal conditions of diet and health there is always available an excess of taurine so that the normal determining factor is the cholic acid radical. Depending upon the available supply of cholic acid there is a high or low output of bile acids in fistula bile. It is evident that certain foods favor a high bile acid output, and presumably furnish considerable amounts of cholic acid in their metabolic history. Much more data on this point will be furnished in other publications.

SUMMARY.

When moderate amounts of bile are given by mouth (less than 1.8 gm. of taurocholic acid) about 90 per cent of the contained taurocholic acid is excreted in the first 4 hours.

Larger amounts of concentrated bile (8.0 to 11.0 gm. of taurocholic acid) may prolong the cholagogue action for many hours or even days.

A large amount of concentrated bile given with sugar causes a very high concentration of bile acids in the bile excreted (7 to 9 per cent). There may even be an absence of cholagogue action. This may represent the maximum effort of the liver cell to concentrate bile acids in bile.

Taurocholic acid and sodium taurocholate given by mouth have the familiar cholagogue action and a large amount will appear in the bile fistula bile (40 to 80 per cent) within 4 to 6 hours, depending upon the dose given.

Taurine intravenously has no effect on the excretion of bile acids.

Taurine plus cholic acid by mouth causes a marked increase in bile secretion and bile acid output—as much as does taurocholic acid itself.

Cholic acid by mouth usually causes a distinct cholagogue effect. Cholic acid fed during long periods of fasting gives a minimal output of bile acids, but fed during full diet periods gives a maximal output of bile acids. This reaction probably depends upon the available supply of taurine, which is much reduced after fasting, but is abundantly available during full diet periods.

We are indebted to Dr. C. L. A. Schmidt for a large amount of taurine which was prepared in his laboratory.

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THE METABOLISM OF BILE ACIDS.

IV. ENDOGENOUS AND EXOGENOUS FACTORS.

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This paper gives the results of experiments to show the excretion of bile acids during periods of fasting, of sugar feeding, and of standard diets. The total daily urinary nitrogen excretion is given in some experiments to show that the output of bile acids runs an interesting parallel to the body's endogenous nitrogen metabolism. Limited diets may profoundly influence the curve of bile acid excretion, and the nitrogenous portion of the diet is most important. It is obvious that certain meat proteins added to the diet profoundly modify the excretion of bile acids. So there seems to be an interesting relationship between the metabolism of meat proteins from the food and of bile acids excreted in the bile. It is at least possible that a similar relationship may hold for the tissue proteins of the body and the bile acid excretion. Much more experimental data must be submitted but the experiments outlined below make certain fundamental points quite clear.

Bidder and Schmidt (1) followed the solid constituents of the bile and concluded that there was an increase on a meat diet. Spiro (3) followed the sulfur in the food and in the bile and found that increasing amounts of meat increased the sulfur excreted, but not proportionately. He also stated that there was a continuous sulfur excretion during fasting, but at a much lower level than when fed. Also, feeding carbohydrates decreases the amount of sulfur excreted. These experiments are in accord with our results.

Kunkel (2) followed the sulfur partition in a single day and found that the sulfur increased in the bile on starvation at one time, and not at another. Bread and milk decreased the sulfur while various amounts of meat did not have any effect at all. Twice that amount of blood by stomach decreased the bile sulfur. The dog lived only about a month after the experiment, so that very little importance can be attached to these experiments.

EXPERIMENTAL.

These dogs were kept on the usual routine already described for the previous articles of this series. The experiments given in Tables XXXI to XXXIV show the urinary nitrogen excretion

TABLE XXXI.

*Sugar Feeding.**Dog 17-151. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	
Mar. 18	13	0.815	10.59	389	38.4		43.0	75 gm. of cane sugar, 50 gm. of glucose given daily by stomach tube.
" 19	16	0.618	9.88	363	69.5	4.76	40.7	Hb. 125 per cent. R. B. C. 6,840,000.
" 20	11	0.972	10.69	392	42.0	3.05	40.8	
" 21	10	0.802	8.02	294	20.9	3.16	39.7	No bile exclusion in this experiment.
" 22						3.89	38.7	
" 23	13	0.780	10.14	372	36.6	2.71	39.0	75 gm. of cane sugar, 100 gm. of glucose given daily by stomach tube.
" 24	12	0.482	5.78	212	37.6	3.33	38.4	
" 25	11	0.556	6.11	224	21.0	3.92	38.5	
" 26	15	0.379	5.68	209	26.1	3.42	38.0	
" 27	30	0.236	7.08	260	14.6	3.41	37.9	
" 28	20	0.279	5.58	205	29.2	3.17	37.25	Hb. 138 per cent. R. B. C. 7,670,000.
" 29	19	0.472	8.98	329	30.8	3.11	37.0	
Average.....			8.05	295		3.45		86 mg. of bile acid per 1.0 gm. of urinary nitrogen.

per 24 hour periods. In these experiments the dogs were kept at all times in standard metabolism cages arranged for complete collection of the urine with elimination of the feces. Diarrhea was never present and no fecal nitrogen is included in these figures. The night bile of course was included in urine collections, but the nitrogen concerned is constant and rarely exceeds 0.2 gm.

per 24 hours. The dogs were catheterized at the same hour each day, weighed, and given the sugar solutions or water by stomach tube. The cage was then washed out and the washings added to the cage urine, bladder urine, and bladder washings, which were made up to a unit volume. Duplicate specimens were analyzed by the Kjeldahl method and the total nitrogen calcu-

TABLE XXXII.

*Fasting.**Dog 17-151. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	
April 23	38	0.513	19.50	716	13.2		40.7	No bile exclusion in this experiment.
" 24	18	0.510	9.18	337	41.3	2.24	38.6	Hb. 132 per cent.
" 25	15	0.704	10.56	388	33.9	4.14	37.4	R. B. C. 6,800,000.
" 26	15	0.508	7.62	279	24.9	3.97	36.6	
" 27	11	0.615	6.76	248	15.2	4.20	36.0	Set up 3 hours.
" 28	27				16.0	4.62	34.5	" " 3 "
" 29	25	0.543	13.68	502	13.1	4.20	35.0	
" 30	15	0.641	9.61	352	12.0	3.83	34.5	
May 1	18	0.711	12.78	468	12.5	3.55	34.13	
" 2	16	0.899	14.39	528	9.5	3.78	33.56	
" 3	15	0.676	10.15	373	11.8	3.97	33.13	Hb. 130 per cent.
								R. B. C. 7,235,000.
Average.....			11.42	419		3.85	109 mg. of bile acid per 1.0 gm. of urinary nitrogen.	

lated. One of the dogs (15-22, Table XXXIV) has an obstruction in his urethra which makes catheterization impossible. The regular 24 hour collections were made as usual, and the average of several days will correct for the daily variations which are in part due to variable amounts of bladder urine retained on different days.

Tables XXXI and XXXII are to be compared, as these experiments were performed upon the same healthy, vigorous dog under

identical experimental conditions. The amount of sugar given in the first experiment was not large for a dog of this size, and was increased from 125 gm. per day to 175 gm. during the last half of the experiment. It may be merely a coincidence, but during the second period of higher sugar intake the bile acid output fell somewhat. This dog averaged 3.45 gm. of urinary nitrogen per day and 295 mg. of taurocholic acid per 6 hours. The 6 hour amount of taurocholic acid per gm. of daily nitrogen is therefore 86 mg.

When this dog is put on fasting after a suitable resting period with liberal mixed diet we note a higher urinary nitrogen output, 3.85 gm. per day and 419 mg. of taurocholic acid per 6 hours. The amount per gm. of 24 hour nitrogen is therefore 109 mg. taurocholic acid per 6 hour period. As the endogenous urinary nitrogen excretion rises with fasting, as compared with sugar feeding, we note a parallel or slightly greater rise in the output of taurocholic acid.

We wish to point out an interesting fact in Tables XXXII and XXXIV. The first day in each table gives a remarkably high taurocholic acid figure, and this may not be clear until one remembers that a full mixed diet preceded this fasting period. The first day's bile acid output results from the mixed diet of the preceding day, but it is significant that after the first 24 hours the base line is reached and maintained. This is somewhat different from the basal nitrogen excretion, which does not reach its lowest level on fasting until the third or fourth day.

Tables XXXIII and XXXIV are like the preceding two experiments and correspond in almost every detail. These two experiments were done on two different dogs of approximately the same weight. Both were in excellent condition. The fasting dog of course shows a higher output of urinary nitrogen and also of taurocholic acid per 6 hour period. The fasting dog shows a urinary nitrogen of 3.76 gm. per 24 hours and taurocholic acid 407 mg. per 6 hours. The sugar fed dog presents a urinary nitrogen of 3.23 gm. per 24 hours and taurocholic acid 318 mg. per 6 hours. The amount of 6 hour taurocholic acid per gm. of 24 hour nitrogen is almost identical, 108 mg. in fasting and 98 mg. with sugar.

It seems sufficiently clear that there is a close relationship

between the endogenous nitrogen metabolism and the excretion of taurocholic acid in bile fistula bile. During fasting periods we may assume that more body protein is broken down and more taurocholic acid results from this process. We may assume that

TABLE XXXIII.
Sugar Feeding.
Dog 18-23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	
Mar. 18	52	0.204	10.60	389	26.2		33.3	75 gm. of cane sugar, 50 gm. of glucose given daily by stomach tube.
" 19	27	0.329	8.88	326	17.4	4.51	31.75	Hb. 118 per cent.
" 20	31	0.324	10.04	368	15.6	3.72	31.06	R. B. C. 6,130,000.
" 21	17	0.507	8.62	316	10.1	3.08	30.75	No bile exclusion in this experiment.
" 22	20	0.414	8.28	304	13.9	3.88	29.19	
" 23	22	0.288	6.33	232	10.7	3.22	29.75	75 gm. of cane sugar, 75 gm. of glucose given daily by stomach tube.
" 24	17	0.512	8.70	319	14.2	2.04	29.56	
" 25	23	0.394	9.06	333	22.0	3.64	29.44	
" 26	26	0.325	8.45	310	11.3	3.13	28.0	
" 27	33	0.194	6.40	235	12.6	3.19	28.56	
" 28	20	0.363	7.26	266	10.4	2.74	27.38	Hb. 120 per cent.
" 29	34	0.332	11.30	415	14.2	2.38	27.06	R. B. C. 7,895,000.
Average.....			8.66	318		3.23		98 mg. of bile acid per 1.0 gm. of urinary nitrogen.

sugar feeding enables the body to conserve its protein at the source, or enables the body to conserve its protein end-products and reconstruct these into body cells. When less bile acids are excreted during sugar periods we may wish to assume some such conservation of bile acid or its parent substance for other uses in

body metabolism. Refer also to Table XL, where there is even more conservation of bile acids or substances from which they are derived.

Fasting does not decrease the bile acid output to a remarkably low level. Sugar feeding causes a drop of bile acid excretion below the fasting level. These two points emphasize the fact

TABLE XXXIV.

Fasting.
Dog 15-22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	
April 23	50	0.668	33.40*	1,225*	21.8		34.7	Dog not catheterized during experiment.
" 24	20	0.602	12.04	442	20.9	5.71	33.0	Hb. 150 per cent.
" 25	27	0.470	12.68	465	32.9	3.75	32.38	R. B. C. 6,390,000.
" 26	18	0.486	8.77	322	21.9	3.16	31.5	No bile exclusion in this experiment.
" 27	28	0.536	15.00	550	4.4	2.91	31.4	
" 28	27	0.552	14.90	547	10.3	4.59	30.56	Set up 3 hours.
" 29	22	0.493	10.85	398	4.2	3.58	30.20	" " 3 "
" 30	20	0.404	8.09	297	22.6	2.94	29.63	
May 1	17	0.505	8.58	315	26.6	3.19	29.20	
" 2	12	0.744	8.93	328	16.3	4.03	28.70	
Average.....			11.09	407		3.76	108 mg. of bile acid per 1.0 gm. of urinary nitrogen.	

* Not included in average.

that there is an important endogenous factor in the bile acid metabolism.

Tables XXXV and XXXVI are identical in practically every respect and show a remarkable parallelism between the food intake nitrogen and the output of taurocholic acid. It will be shown later that this reaction depends in part upon the type of food protein. The first dog (Table XXXV) was in perfect condition during the entire experiment, maintained a constant weight,

TABLE XXXV.

*Nitrogen in Food and Bile Acid Excretion.
Dog 17-151. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Jan. 7	43	0.403	17.32	636	21.4	38.0	Diet 375 gm. of cracker meal, 90 gm. of beef heart containing 100 calories and 0.5 gm. of nitrogen per kilo.
" 8	30	0.575	17.25	633	23.0	38.0	Hb. 135 per cent. R. B. C. 7,576,000.
" 9	49	0.246	12.05	442	24.7	38.0	
" 10	32	0.505	16.16	593	10.6	38.5	
" 11	28	0.311	8.70	319	6.3	38.5	
" 14	20	0.680	13.60	499	20.8	38.5	
" 15	65	0.280	16.90	620	20.1	39.0	No bile exclusion in this experiment.
" 16	28	0.766	21.44	787	18.5	39.0	
" 17	37	0.538	19.90	730	19.9	39.3	
" 18	17	0.785	13.35	490	16.0	38.8	Diarrhea.
" 21	16	0.604	9.66	354	18.9	39.0	
" 22	23	0.591	13.59	499	18.4	39.0	
Average.....				14.99	550	18.2	Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	67	0.415	27.80	1,020	23.6	39.2	696 gm. of beef heart, 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	50	0.460	27.00	994	14.8	39.5	
" 25	21	0.500	10.50	386	1.8	39.0	
" 28	37	0.675	24.97	918	3.9	39.3	
" 29	25	1.016	25.40	934	1.7	39.0	
" 30	32	0.896	28.73	1,055	2.8	39.3	
" 31	37	0.733	27.12	998	7.7	39.0	
Feb. 1	44	0.729	32.09	1,177	20.7	39.0	
" 4	51	0.645	32.89	1,206	8.0	39.0	
" 6	18	1.083	19.49	715	27.6	39.0	
" 7	40	0.871	34.84	1,279	7.7	38.8	
" 8	35	0.870	30.45	1,118	10.4	39.0	
Average.....				26.77	982	10.9	Diet 1.0 gm. of nitrogen per kilo.

TABLE XXXVI.

*Nitrogen in Food and Bile Acid Excretion.
Dog 18-23. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Jan. 7	42	0.272	11.02	405	40.9	33.0	Diet 325 gm. of cracker meal, 80 gm. of beef heart containing 0.5 gm. of nitrogen and 100 calories per kilo.
" 8	62	0.158	9.79	359	27.0	33.3	
" 9	72	0.073	5.22	191	25.0	33.0	Hb. 115 per cent. R. B. C. 7,024,000.
" 10	67	0.206	13.80	506	29.4	33.0	
" 11	69	0.158	10.93	401	17.6	32.8	No bile exclusion in this experiment.
" 14	67	0.143	9.58	453	20.8	33.0	
" 15	76	0.088	6.68	245	26.4	33.0	
" 16	56	0.211	11.81	433	14.6	33.5	
" 17	74	0.057	4.21	154	18.1	33.1	
" 18	70	0.184	12.85	472	12.2	33.3	
" 21	75	0.202	15.13	555	18.3	33.5	
" 22	68	0.226	15.40	566	19.9	33.5	
Average.....			10.53	395	22.5		Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	79	0.3431	27.1	994	15.2	33.8	602 gm. of beef heart, 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	77	0.246	18.93	685	18.8	33.6	
" 25	82	0.217	17.77	552	16.7	33.0	
" 28	43	0.392	16.85	618	11.0	32.0	350 gm. not eaten. Diarrhea.
" 29	58	0.416	24.12	885	9.1	31.8	250 " " " "
" 30	55	0.489	26.89	986	22.0	30.5	110 " " " "
" 31	70	0.424	29.68	1,089	20.7	30.5	30 " " " "
Feb. 1	68	0.380	25.84	948	18.2	30.3	
" 4	80	0.312	24.96	916	18.1	29.5	Diarrhea.
" 5	60	0.395	23.70	870	14.6	29.0	" Noon meal omitted.
" 6	48	0.317	15.21	558	8.4	29.5	
" 7	74	0.213	15.76	578	14.1	29.0	
" 8	47	0.307	14.42	529	11.5	28.5	
Average.....			21.63	794	15.2		Diet 1.0 gm. of nitrogen per kilo.

and ate all food. The bile pigment figures are included and show remarkable and inexplicable fluctuation, particularly with the beef heart diet.

The taurocholic acid output is quite uniform each day with occasional fluctuations. The average daily output of bile acid per 6 hour period shows a very remarkable increase when the dog was suddenly changed from the diet rich in carbohydrate (0.5 gm. of nitrogen per kilo) to the beef heart diet (1.0 gm. of nitrogen per kilo). The increase in bile acid corresponds to the increase in food nitrogen—that is, about 100 per cent increase. The sharp rise in taurocholic acid following the change to a rich protein diet (beef heart) is well shown in both these experiments. The reaction even goes above the average figures of taurocholic acid output on the very first day of rich protein diet.

The second dog (Table XXXVI) shows a general reaction which is in every respect similar to the preceding experiment but for some diarrhea in the beef heart period. Associated with this was some loss of weight and appetite. These abnormal factors, however, did not influence the uniform reaction to the change in food protein. Additional data which confirm these experiments will be found in Paper V of this series.

Tables XXXVII and XXXVIII add some interesting data to that of the preceding experiments. In both these experiments the food nitrogen was decreased to 0.25 gm. per kilo by decreasing the cracker meal and replacing the beef heart with fat. The caloric value was held unchanged at 100 calories per kilo. One dog (Table XXXVII) showed practically no reaction to this change in diet and excreted almost the same amount of taurocholic acid as formerly upon a diet of 0.5 gm. of nitrogen per kilo. The other dog (Table XXXVIII) showed a decided drop in excretion of taurocholic acid but not to one-half the output on the diet containing 0.5 gm. of nitrogen per kilo. The level of bile acid output on this cracker meal-fat diet approaches the fasting excretion level of bile acid. This shows at once that there is no hard and fast parallel between the nitrogen intake and bile acid excretion, but it is clear that there is an important exogenous factor.

The mixed diet periods show a level of bile acid excretion which is below that of the beef heart diet. The elements in the

TABLE XXXVII.

*Nitrogen in Food and Bile Acid Excretion.**Dog 17-151. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Feb. 11	32	0.525	16.80	617	38.8	40.0	262 gm. of cracker meal, 10 gm. of butter, 30 gm. of lard, 105 gm. of cane sugar, 0.25 gm. of nitrogen, and 100 calories per kilo.
" 12	30	0.536	16.08	590	28.2	39.5	
" 13	11	0.602	6.62	243	10.4	39.8	Hb. 125 per cent. R. B. C. 6,240,000.
" 14	32	0.425	13.60	499	20.6	40.0	
" 15	24	0.527	12.66	465	20.9	40.0	
" 18	32	0.385	12.32	452	10.0	40.0	No bile exclusion in this experiment.
" 19	20	0.493	9.86	362	13.2	40.0	
" 20	45	0.345	15.52	570	14.9		
" 21	23	0.695	15.98	586	5.2		
Average.....				13.27	487	18.0	Diet 0.25 gm. of nitrogen per kilo.
Mar. 5	61	0.534	32.55	1,196	16.6	42.5	Mixed diet.
" 6	48	0.662	31.79	1,167	21.5	41.8	
" 7	80	0.290	23.19	852	36.9	42.5	Hb. 118 per cent. R. B. C. 6,350,000.
" 8	32	0.695	22.25	818	10.1	43.0	
" 11	38	0.286	10.86	399	21.9	41.5	
" 12	30	0.771	23.14	850	3.9	43.0	
" 13	28	0.786	22.00	809	11.3	42.5	
" 14	40	0.616	24.64	906	5.8	43.5	
" 15	22	0.691	15.20	559	12.6	44.0	
Average.....				22.85	839	15.6	Mixed diet.

TABLE XXXVIII.

*Nitrogen in Food and Bile Acid Excretion.
Dog 18-23. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Feb. 11	48	0.231	11.08	407	18.4	30.3	30 gm. of lard, 59 gm. of cane sugar, 198 gm. of cracker meal, 10 gm. of butter, 0.25 gm. of nitrogen, and 100 calories per kilo.
" 12	43	0.174	7.47	274	16.1	29.5	
" 13	68	0.100	6.80	253	16.6	29.5	Hb. 115 per cent. R. B. C. 7,928,000.
" 14	58	0.027	1.60	58	18.2	29.3	
" 15	63	0.129	8.10	297	18.4	29.3	No bile exclusion in this experiment.
" 18	35	0.114	3.97	145	13.4	29.0	
" 19	64	0.197	12.64	464	11.1	29.2	
" 20	55	0.172	9.48	348	19.1		
" 21	51	0.085	4.34	159			
Average.....			7.27	267	16.4		Diet 0.25 gm. of nitrogen per kilo.
Mar. 5	75	0.241	18.06	664	31.8	32.0	Mixed diet.
" 6	64	0.249	15.93	585	36.3		
" 7	85	0.173	14.73	541	37.0	33.3	Hb. 110 per cent. R. B. C. 6,290,000.
" 8	81	0.210	17.04	628	37.5	34.0	
" 11	91	0.200	18.22	670	27.9	32.8	
" 12	69	0.184	12.72	467	29.0	34.0	
" 13	82	0.324	26.60	978	25.3	35.0	
" 14	60	0.300	18.01	662	27.6	35.5	
" 15	82	0.307	25.14	923	29.5	34.8	
Average.....			18.50	679	31.3		Mixed diet.

mixed diet are of course variable. This diet is a mixture of kitchen scraps—bones, meat, bread, potato, rice, etc.

Table XXXIX shows a third dog which was placed upon these same diets containing 0.5, 1.0, and 0.25 gm. of nitrogen per kilo. This dog remained in perfect condition during the entire experiment and adds confirmatory data to the other experiments. This bile fistula dog has been under observation for 3 years, and it is known that there is a small communication between the common duct and duodenum. This allows a small amount of bile to enter the duodenum when the fistula is not draining freely, for example at night. This dog shows only a 50 per cent rise in bile acid output when the diet is changed from 0.5 to 1.0 gm. of nitrogen per kilo. Further change in diet from 1.0 to 0.25 gm. of nitrogen per kilo causes a fall from 1,262 mg. of taurocholic acid to 684 mg. per 6 hour period. These fluctuations are not proportional to the nitrogen content of the diets, but it is obvious that a rise in the food nitrogen intake does cause a rise in the taurocholic acid excretion and *vice versa*.

Table XL gives the data on two experiments which show the influence of a preceding period of fasting upon subsequent excretion of bile acids with a standard diet of 0.5 gm. of nitrogen per kilo. It is to be recalled that these same two dogs on a previous occasion showed an output of bile acids on this same diet which was much higher (550 and 875 mg. of taurocholic acid per 6 hour period—Tables XXXV and XXXIX). Compare with this high output the low excretion in Table XL on the same diet (376 and 419 mg. of taurocholic acid per 6 hour period). The only factor which can explain this difference is the preceding fasting period of 10 and 11 days. This low level of excretion is actually that of the fasting period or even lower. It is evident that the fasting period has caused a changed reaction in the body so that much less bile acid is permitted to escape in the bile. It may be a correct assumption that some of the material which under usual diet conditions goes to form the bile acid fraction is deviated for other uses in the body.

This reaction must be kept in mind whenever any diet experiments are planned for these bile fistula dogs. For certain experiments it might be assumed that a preliminary period of fasting might give an ideal simple base line from which to estimate the change brought about by a given diet. But the reaction may be

TABLE XXXIX.

*Nitrogen in Food and Bile Acid Excretion.**Dog 15-22. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Jan. 7	13	0.835	10.85	398	10.0	32.0	325 gm. of cracker meal, 75 gm. of beef heart. 100 calories and 0.5 gm. of nitrogen per kilo.
" 8	20	0.992	19.84	728	3.5	32.3	
" 9	32	0.662	21.18	778	3.3	32.3	
" 10	26	0.591	15.36	551	3.6	32.5	No bile exclusion in this experiment.
" 11	38	0.373	14.17	520	7.3	32.3	
" 14	35	1.071	37.48	1,375	8.5	32.3	Large amount not eaten.
" 15	34	0.751	25.53	936	4.1	32.0	100 gm. not eaten.
" 16	14	0.920	12.88	473	3.4	32.0	100 " " "
" 17	46	0.630	28.98	1,065	6.4	32.5	150 " " "
" 18	43	0.822	35.35	1,297	7.5	32.0	
" 21	60	0.759	45.54	1,670	4.2	32.3	25 " " "
" 22	42	0.453	19.00	698	9.5	32.0	
Average.....		23.84		875	5.9		Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	38	0.915	34.77	1,277	5.3	32.0	571 gm. of beef heart containing 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	45	0.750	33.75	1,238	8.6	31.5	
" 25	62	0.700	43.40	1,593	5.4	31.0	
" 28	35	0.566	19.81	727	24.2	30.5	Diarrhea.
" 29	22	1.400	30.80	1,130	10.3	30.8	
" 30	30	1.730	51.90	1,905	7.8	31.0	
" 31	46	1.100	50.60	1,855	11.2	30.8	
Feb. 1	28	1.394	29.03	1,065	6.8	30.8	
" 4	29	1.131	32.80	1,205	13.6	31.0	
" 5	39	1.015	39.58	1,453	6.0	30.8	Noon meal omitted.
" 6	12	1.896	22.75	835	6.3	31.0	
" 7	42	0.698	29.31	1,075	6.6	30.5	
" 8	29	0.980	28.42	1,042	11.6	30.3	
Average.....		34.37		1,262	9.5		Diet 1.0 gm. of nitrogen per kilo.

TABLE XXXIX—*Concluded.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Feb. 11	29	1.105	32.05	1,175	11.6	32.0	209 gm. of cracker meal, 10 gm. of butter, 30 gm. of lard, 65 gm. of cane sugar, 0.25 gm. of nitrogen and 100 calories per kilo.
" 12	23	0.654	15.04	550	16.0	31.3	
" 13	47	0.403	18.95	696	8.4	32.0	
" 14	23	0.633	14.56	534	36.0	31.5	Hb. 124 per cent. R. B. C. 5,632,000.
" 15	48	0.658	31.58	1,160	10.8	31.8	
" 18	45	0.296	13.32	488	24.3	30.5	
" 19	28	0.331	9.26	340	14.6	30.5	
" 20	12	1.410	16.92	620	13.0	31.0	
" 21	17	0.922	14.97	599	17.2	31.3	
Average.....			18.50	684	16.8		Diet 0.25 gm. of nitrogen per kilo.

very different toward the same diet factor depending upon whether a fasting period or a carbohydrate diet period had preceded. To get a complete understanding of a single diet factor it will be necessary to observe any change in the bile acid excretion curve which may be associated with the administration of any such substance after short fasting periods as well as after carbohydrate or high protein diet periods.

Table XLI gives data to indicate that the formation of bile acids depends in part upon the functional capacity of the liver. The Eck fistula liver is produced by an anastomosis between the portal vein and vena cava and a ligature on the portal vein above this anastomosis which limits the blood supply of the liver to the hepatic artery and deviates all of the portal blood directly into the vena cava. The Eck fistula liver is known to be smaller than normal, to exhibit a moderate degree of fatty degeneration, to show a marked decrease in production of bile pigments (Whipple and Hooper, 4), and to present a distinct impairment of its normal capacity to

TABLE XL.

Nitrogen in Food and Bile Acid Excretion after Fasting Period.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.				
17-151	Average 10 days fasting				11.8	433		
	1918 May 4	cc.	mg.	mg.	mg.	mg.	lbs.	Diet 330 gm. of cracker meal, 75 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo. Hb. 130 per cent. R. B. C. 7,235,000.
		37	0.454	16.80	616	11.0	32.6	
	" 6	19	0.349	6.64	224	1.6	36.25	
	" 7	20				2.4	35.5	
	" 8	27	0.367	9.91	364	3.8	35.8	
	" 9	25	0.350	8.75	321	6.1	34.7	
	" 10	24	0.407	9.77	359	6.7	35.0	
Average				10.37	376			Diet 0.5 gm. of nitrogen per kilo.
15-22	Average 11 days fasting.....				11.09	407		
	May 6	21	0.587	12.32	452	2.9	29.2	Diet 285 gm. of cracker meal, 60 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo. Hb. 125 per cent. R. B. C. 7,335,000.
	" 7	32				5.6	29.5	
	" 8	36	0.353	12.70	466	9.4	29.2	
	" 9	29	0.365	10.58	388	6.1	29.0	
	" 10	24	0.421	10.10	371	4.2	29.2	
Average				11.42	419			

excrete phenoltetrachlorphthalein (Whipple, Peightal, and Clark, 5). We may assume that the Eck fistula liver is functionally deficient. The observations in Table XLI harmonize with those

TABLE XLI.
*Nitrogen in Food and Bile Acid Excretion. Eck Fistula.
Dog 16-15. Simple Bile and Eck Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
Jan. 7	16	0.314	5.03	183	4.5	20.5	Diet 200 gm. of cracker meal, 55 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo.
" 8	25	0.258	6.45	236	5.7	20.0	Hb. 99 per cent. R. B. C. 6,240,000.
" 10	12	0.219	2.62	96	1.9	19.8	Not eating well.
" 11	16	0.144	2.30	84	5.3	19.8	" " "
" 14	12	0.155	1.86	68	3.3	20.0	
" 15	6	0.116	0.69	25		20.2	
" 16	10	0.141	1.41	52	1.6	20.7	
" 17	14	0.330	4.62	169	3.2	19.8	
" 18	21	0.273	5.73	210	3.7	20.3	50 gm. not eaten.
" 21	20	0.310	6.20	227	2.1	20.0	
" 22	20	0.212	4.24	155	2.1	20.0	
Average.....			3.74	137	3.34	Diet 0.5 gm. of nitrogen per kilo.	
Jan. 24	30	0.447	13.41	492	3.7	19.8	Diet 355 gm. of beef heart, containing 1.0 gm. of nitrogen and 100 calories per kilo, 1 gm. of yeast twice a day.
" 25	17	0.534	9.07	333	0	20.8	

outlined above and show that an Eck fistula dog upon a standard diet will excrete not over one-half the normal amount of bile acids.

Compare this dog (Table XLI), weight 20 lbs. and output on standard diet of 137 mg. of taurocholic acid per 6 hour period, with the dog of Table XXXV, weight 40 lbs. and output on the same

standard diet of 550 mg. of taurocholic acid per 6 hour period. Also compare with dog of Table XXXIX, weight 33 lbs. and output on the same standard diet of 875 mg. of taurocholic acid per 6 hour period.

When the Eck fistula dog was changed to a rich protein diet we observe a considerable jump in the output of bile acids, as in the other dogs. It is not possible to keep an Eck fistula dog on a pure beef heart diet for any length of time without precipitating the characteristic Eck fistula intoxication which usually results in death.

DISCUSSION.

There is sufficient experimental data given above to make the point that both endogenous and exogenous factors are concerned in the metabolism of bile acids. There is a reasonably constant output of taurocholic acid during fasting periods, and this output may be somewhat diminished by administration of pure carbohydrate. The relative diminution of urinary nitrogen and taurocholic acid excretion may show a certain similarity under these experimental conditions. This may indicate a certain relationship between the metabolism of the body protein and the production of taurocholic acid. There is obviously a very important endogenous factor in the metabolism of bile acids.

It is equally clear that there is an important relationship between the output of taurocholic acid and the intake of certain food proteins. On certain diets a uniform level of bile acid excretion may be observed for days and a sudden shift to a similar diet containing double the amount of food nitrogen may cause a sudden doubling of bile acid excretion. This fact comes out clearly in several experiments in this paper but we wish to refer to additional evidence submitted in Paper V of this series. It is certain that some food proteins act very differently from others as to their value in modifying the bile acid excretion.

It is to be noted that the increased excretion of bile acids appears very promptly when the diet is changed to beef heart. It may be that the formation of bile acids by the liver cell is an automatic response to the proper stimulus, just as these cells respond to a protein digestion stimulus by urea formation. In the case of urea formation we know that even in the greatest food

shortage emergency the liver will form urea from amino-acids, even though the body may need all the food amino-acids, for example, after long periods of fasting. In the case of taurocholic acid we have shown that long periods of fasting followed by high protein feeding will show little rise in the taurocholic acid output contrary to what we might expect if this substance was purely a waste product to be eliminated from the split products of protein digestion. The body evidently conserves the taurocholic acid or its parent substance under certain conditions, perhaps for use elsewhere in the body in the reconstruction of its depleted body protein.

It is generally accepted that bile acids are dependent upon the normal liver cell function for their production. There has been little dispute in medical literature concerning this point. Yet there is very little available direct proof of this statement if one wished to argue that the bile acids were formed elsewhere in the body and eliminated in the bile. For example there is convincing evidence that bile pigments may be formed outside of the liver and excreted in the bile secondarily (Whipple and Hooper).

We have evidence in the Eck fistula experiment given above that bile acid output will be much subnormal in the Eck fistula liver which is functionally subnormal. This indicates that in a general way the bile acid output may fall with impaired functional capacity of the liver cell. Other experiments in which the liver is injured by chloroform and other poisons give similar results which will be published in the near future. All this evidence gives some direct and positive proof that the bile acids are actually produced by liver cell activity.

SUMMARY.

There is a uniform excretion of taurocholic acid in the bile fistula dog during fasting periods.

There is a uniform and slightly lower excretion of taurocholic acid in the same dog during similar periods of carbohydrate (sugar) feeding. This fall in taurocholic acid excretion is much like the fall in urinary nitrogen excretion under the same conditions.

There is an important endogenous factor in the bile acid metabolism and this may concern the body protein metabolism.

The output of bile acid in bile fistula bile may be influenced at will by suitable control of the diet. Meat protein seems to be of the greatest importance and a pure meat diet will give the highest output of bile acid per 6 hour period.

There is an important exogenous factor in bile acid metabolism which is concerned especially with the food protein.

After a long fasting period the bile fistula dog will not react to a high protein diet with the usual high bile acid output. There is evidently a deviation of certain precursors of the bile acid to serve other purposes in the body—perhaps to supply some important substances relating to body protein which have been depleted by the fasting period.

A functionally deficient liver (Eck fistula) produces less than one-half the normal amount of bile acid during a standard diet period. This is direct evidence (of which there has been little available) that the bile acids are formed essentially by liver cell activity.

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THE METABOLISM OF BILE ACIDS.

V. CONTROL OF BILE INGESTION AND FOOD FACTORS.

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The experiments tabulated below serve two purposes. They furnish additional data to make absolutely certain that the amount of bile acid excreted can be increased with the increase of meat protein nitrogen in the diet. These experiments in addition show that absolute exclusion of every drop of bile by mouth does not modify the excretion of bile acids under the conditions of the experiment. It was necessary to submit these control experiments because Stadelmann, who has done exceptional experimental work in this field, has stated that bile exclusion will cause a fall of bile acid excretion. He assumes therefore that a muzzle at night is necessary for accurate work, and that the bile fistula dogs lick sufficient bile from their fistulas to modify the following day's excretion of bile and bile acids.

Our dogs are set up each morning after a period of exercise followed by a 30 minute period of free drainage before collections are started. After the collection period of 6 hours the dogs are allowed to exercise in the yard before being fed in their cages. They are kept during the night in large cages of wire mesh beneath which are pans for the collection of excreta. The dogs, of course, during the night drain bile from their fistulas into the pans, but no bile collects where the dogs have access to it because the wire mesh retains no fluids. The dogs usually lick their fistulas when the collection is finished but it seems to be due in part to the fact that the skin itches where it is in contact with the abdominal binder. These dogs rarely lick bile from their fistulas in the early morning and at this time the flow is at a minimum. We feel that the experiments given make it quite clear that under

this laboratory routine the amount of bile which may be obtained by a given dog licking its own fistula is not sufficient to modify the excretion curve of bile acids.

We cannot attempt to explain Stadelmann's results, but two factors may be concerned. It is well known that dogs which have had bile fistulas for many months or years do not lick their fistulas as much as do dogs who are less accustomed to the bile fistula. One of our dogs has been under observation with a bile fistula for $3\frac{1}{2}$ years. Furthermore, in our cages, as stated above, bile could not accumulate during the night where the dog had access to it at any time, as the bile flowed through the mesh cage floor. If bile accumulated on the floor of a room or cage it is very probable that a dog would lick some of this bile in the morning and obtain a sufficient amount to make a decided difference in the output of bile acids.

It can be deduced from the experiments given in Paper III that moderate amounts of bile given late in the afternoon will not influence the output of bile acids on the following day. Bile given in the forenoon will cause a cholagogue action lasting several hours, but usually a rapid elimination of the excess of bile acids within 6 hours—at least 80 to 90 per cent elimination within this time.

EXPERIMENTAL.

Bile exclusion in these experiments indicates that the dog in question was unable to gain access at any time to any bile either from its own fistula or elsewhere. During the routine 6 hour collection all the bile is collected in a small rubber bag. At the end of the collection a muzzle is put securely on the dog, which is then permitted to run in the yard for a few minutes under careful observation. The dog is then brought in and dressed with its night binder which is worn until the next morning. The muzzle was not worn during the night except in one instance, where the dog tried to chew off the straps of the abdominal binder. The night binder was made of light canvas and fitted to the individual to cover the thorax and abdomen completely. Anteriorly it was held from slipping backward by soft webbing which encircles the neck and fore legs. The binder was held about the abdomen by soft webbing or straps. A large gauze pad was

placed over the bile fistula and served to absorb all the night bile. With this routine we are absolutely certain that no bile was ingested at any time during periods of "bile exclusion." The animals were comfortable and maintained their usual conditions of diet and activity.

Tables XLII, XLIII, and XLIV are to be considered as a unit. These three experiments were done at the same time under identical conditions, and the results are remarkably uniform. They are to be compared with Tables XXXV, XXXVI, and XXXIX in Paper IV of this series. In each of the three experiments tabulated (XLII to XLIV) the dog was placed upon a diet of cracker meal and beef heart containing 0.5 gm. of nitrogen and 100 calories per kilo. This diet with complete bile exclusion obtained for 1 week, and the average daily output per 6 hours shows a fairly uniform figure of 400 to 500 mg. of taurocholic acid per 6 hour period. The dog which is slightly heavier shows a slightly greater output.

The three dogs kept on this same diet for the second week were not prevented from licking their own fistulas in the cage during the night. It will be seen that the bile acid output remains about the same—it is actually somewhat less during this second week, 340 to 440 mg. of taurocholic acid per 6 hours.

During the third week each dog was given the first day with no bile exclusion, the second and third day with complete bile exclusion, and the fourth day with no bile exclusion. Individual fluctuations appear but this week in general agrees with the data of the first 2 weeks.

The fourth week was continued with strict bile exclusion but the diet was changed to a mixture of beef heart and a little cracker meal, giving 1.0 gm. of nitrogen and 100 calories per kilo. The figures from this group of experiments resemble those referred to in Paper IV. The increase in beef heart stimulates the output of bile acids from a level of 400–500 mg. to 700–900 mg. per 6 hours.

Two points are made by these and other experiments. Bile exclusion does not modify the excretion of bile acids under the conditions of our experiments. Certain food proteins in the diet have a marked influence on the excretion of bile acids in bile fistula bile.

TABLE XLII.

*Known Diet With and Without Bile Exclusion.
Dog 18-93. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
Aug. 5	57 ±	0.256	14.59 ±	536 ± *	31.8	Absolute bile exclusion. Diet 330 gm. of cracker meal. 60 gm. o' beef heart = 0.5 gm. of nitrogen and 100 calories per kilo.
" 6	46	0.320	14.70	540	31.5	
" 7	38 ±	0.254	9.66 ±	355 ± *	32.0	
" 8	56	0.238	13.33	489	32.0	
" 9	59	0.255	15.05	552	32.3	
Average.....			13.50	494	Complete bile exclusion.	
Aug. 12	54	0.224	12.08	443	30.8	No bile exclusion.
" 13	61	0.210	12.81	470	31.5	
" 14	58 ±	0.242	14.05 ±	516 ± *	31.5	
" 15	61	0.158	9.64	354	31.3	
" 16	64	0.181	11.58	425	31.3	
Average.....			12.00	442	No bile exclusion.	
Aug. 19	70	0.266	18.62	683	31.0	No bile exclusion.
" 20	71	0.196	13.90	510	30.5	Bile exclusion 12 hours previously.
" 21	64	0.230	14.72	540	30.8	Bile exclusion.
" 23	77	0.185	14.25	523	30.5	No bile exclusion.
Aug. 28	72	0.326	23.48	862	30.0	Absolute bile exclusion. Diet 467 gm. of beef heart, 100 gm. of cracker meal = 1.0 gm. of nitrogen and 100 calories per kilo.
" 29	72	0.293	21.08	774	29.8	
" 30	76	0.335	25.48	934	29.5	
Average.....			23.30	857	Complete bile exclusion.	

* 2-3 cc. of bile lost.

TABLE XLIII.

*Known Diet With and Without Bile Exclusion.
Dog 18-54. Bile Fistula and Splenectomy.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
Aug. 5	65	0.170	11.05	405	27.5	Absolute bile exclusion. Diet 280 gm. of cracker meal, 60 gm. of beef heart = 0.5 gm. of nitrogen and 100 calories per kilo.
" 6	62	0.181	11.23	412	27.8	
" 7	41	0.100	4.10	150	28.0	Binder chewed off night of the 6th.
" 8	47	0.182	8.56	314	27.5	
" 9	79	0.212	16.75	614	27.0	
Average.....			10.33	379		Complete bile exclusion.
Aug. 12	77 ±	0.224	17.25 ±	633	27.3	No bile exclusion. 5 hour collection.
" 13	51	0.140	7.14	262	27.3	
" 14	43	0.172	7.40	271	27.0	
" 15	57	0.115	6.56	241	27.0	
" 16	53	0.126	6.68	245	27.5	
Average.....			9.00	330		No bile exclusion.
Aug. 19	77	0.222	17.10	628	27.5	No bile exclusion.
" 20	76	0.167	12.68	465	27.3	Bile exclusion 12 hrs. pre- viously.
" 21	71	0.187	13.26	486	27.5	Bile exclusion.
" 23	80	0.128	10.24	376	27.3	No bile exclusion.
Aug. 27	46				27.0	Absolute bile exclusion. Diet 417 gm. of beef heart and 100 gm. cracker meal = 1.0 gm. of nitrogen and 100 calories per kilo.
" 28	81	0.288	23.31	856	26.3	
" 29	75	0.311	23.32	856	26.5	
" 30	70	0.380	26.60	976	26.3	Hb. 110 per cent. R. B. C. 5,515,000.
Average.....			24.40	896		Complete bile exclusion.

TABLE XLIV.

*Known Diet With and Without Bile Exclusion.
Dog 18-137. Bile Fistula and Splenectomy.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
Aug. 5	53	0.313	16.58	608	23.0	Absolute bile exclusion. Diet 245 gm. of cracker meal, 45 gm. of beef heart = 0.5 gm. of nitrogen and 100 cal- ories per kilo.
" 6	56	0.264	14.78	542	23.0	
" 7	38	0.365	13.86	509	23.3	
" 8	37	0.250	9.25	340	23.0	
" 9	34	0.198	6.73	247	23.0	
Average.....			12.25	450		Complete bile exclusion.
Aug. 12	56	0.196	10.96	402	22.8	No bile exclusion.
" 13	46	0.266	12.24	449	22.3	
" 14	40	0.214	8.56	314	22.0	
" 15	43	0.129	5.50	202	22.0	
" 16	44	0.198	8.71	320	22.0	
Average.....			9.20	338		No bile exclusion.
Aug. 19	50	0.404	20.20	742	22.0	3 hour collection.
" 20	57	0.292	16.65	611	22.3	Bile exclusion 12 hours pre- viously.
" 21	68	0.188	12.78		22.3	Bile exclusion.
" 23	40	0.099	3.96	145	22.3	No bile exclusion.
Aug. 28					21.8	Absolute bile exclusion. Diet 327 gm. of beef heart and 100 gm. of cracker meal = 1.0 gm. of nitrogen and 100 cal- ories per kilo.
" 29	50	0.335	16.75	611	22.0	
" 30	57	0.376	21.42	786	22.0	Hb. 120 per cent. R. B. C. 5,060,000.
Average.....			19.07	698		Complete bile exclusion.

Tables XLV and XLVI supply more data on bile exclusion and fasting excretion of bile acids. These tables are to be compared with Tables XXXII and XXXIV in Paper IV and the average of these last two tables is added to Tables XLV and XLVI now under consideration. Table XLV shows practically the same output of bile acids per 6 hours whether bile is excluded or not. This is in harmony with all the other experiments given in this and other papers as well as many experiments which are unpublished.

TABLE XLV.
Bile Exclusion—Fasting.
Dog 17-151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
May 27	28	0.693	19.40	712	26.8	41.2	Hb. 120 per cent. R. B. C. 7,520,000.
" 28	24	0.541	12.98	476	23.8		
" 29	19	0.492	9.35	343	9.3	39.0	
" 30	20	0.558	11.20	411	9.0	38.0	
" 31	19	0.653	12.41	455	13.9	37.1	
June 1	23	0.694	15.96	586	10.0	36.5	
Average 6 days ..		13.55		497			Complete bile exclusion.
Average 10 days ..		11.42		419			No bile exclusion. Table XXXII.

Table XLVI presents a single contradiction to all the rest of our published data and taken by itself would seem to indicate that bile exclusion did actually diminish the bile acid output. Such contradictions are not unheard of in experimental work, but we believe this exception to the general rule is in reality explicable. This dog as stated above (Paper IV) is known to have a small opening from his biliary tract into the duodenum. At times he presents remarkable variations in the output of bile acids which stand in contrast to the other dogs whose biliary passages are absolutely separated from the duodenum. Because of this fact we attach little importance to observations on this dog

when the bile is concentrated and viscid, and still less importance to observations which differ from those made upon the other fistula dogs.

TABLE XLVI.

Bile Exclusion—Fasting.
Dog 15-22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	cc.	mg.	mg.	mg.	mg.	lbs.	
May 27	27	0.905	24.30*	892*	3.7	34.5	Hb. 130 per cent. R. B. C. 6,540,000.
" 29	12	0.532	6.38	234	14.6	32.3	
" 30	21	0.435	9.14	335	18.3	31.5	
" 31	16	0.380	6.08	223	9.7	30.9	
June 1	12	0.562	6.74	247	14.6	30.4	
" 2	20	0.086	1.72	63	46.0	29.7	NH ₂ determination repeated with same result.
" 3	16	0.510	8.16	299	24.5	29.4	
Average 6 days ...			6.37	234			Complete bile exclusion.
Average 9 days ...			11.09	407			No bile exclusion. Table XXXIV.

* Not included in average.

SUMMARY.

Complete exclusion of bile ingestion at all times does not modify the excretion of bile acids from day to day. This statement applies to the dogs used in our experiments and to the laboratory conditions under which all this work was done. It is not necessary therefore to take elaborate precautions to prevent any given dog from licking its fistula during afternoon and night resting periods.

These experiments confirm those tabulated in the preceding paper to show that an increase in the meat proteins in the diet will cause a considerable rise in output of taurocholic acid.

THE METABOLISM OF BILE ACIDS.

VI. ORIGIN OF TAUROCHOLIC ACID.

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Taurocholic acid can be easily separated into its two essential constituents, taurine and cholic acid. It has been shown (Paper III) that in the body the two constituent parts can be easily and rapidly united to form taurocholic acid. This holds true whether the two substances are given by mouth or whether cholic acid is given by mouth and taurine intravenously. It is clear that cholic acid and taurine have a strong physiological attraction in the normal body.

Taurine appears to be present in the normal body in a certain excess, judging from cholic acid feeding experiments. Taurine is more abundant in the body, at least more accessible for linkage with cholic acid, during full diet periods than during fasting periods (Paper III). We have also submitted evidence to show that taurine is derived at least in part from the cystine of the food. This is in harmony with the observations of von Bergman (2) and Wohlgemuth (14), who used somewhat inaccurate methods of analysis. It is highly probable that taurine may be derived from other substances under certain conditions but we have no direct proof concerning this point. When the metabolism of taurine is fully understood we may be nearer a complete understanding of the complex bile acid metabolism.

Cholic acid is a substance which defies the investigator, who up to the present time has learned little about its source or usefulness in the body. Cholic acid is of particular interest to us because its supply appears to be the normal limiting factor which determines the bile acid level of excretion. A sufficient supply of taurine in the body seems to obtain under all physiological conditions. There is sufficient evidence to show the important relationship that

exists between the basal nitrogen metabolism and bile acid metabolism. We believe that knowledge concerning the bile acid metabolism will be of value for a complete understanding of the fundamental body protein metabolism.

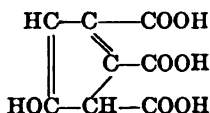
Cholic acid is somewhat complex and its accepted formula is given below. Because of its structural formula many hypotheses have been advanced concerning its origin in the body and its possible relationship to various drugs. Some positive experiments have been reported to indicate a relationship between cholic acid and cholesterol, but these data will not bear careful scrutiny. In the experiments given below we have tested a number of such hypotheses and without exception can report only negative results. It seemed to us of some importance to establish beyond a doubt that cholesterol and allied substances have no influence whatever on the cholic acid metabolism.

Through the work of von Bergman in 1904, and Wohlgemuth in the same year, it was clearly established that some of the taurine of the taurocholic acid came from cystine. Both used the sulfur method in their experiments. Friedman (4) in 1903 had shown that this change could be effected *in vitro*, but von Bergman was the first to show that when cystine is fed to a dog together with sodium cholate on a constant diet, there is a marked increase in the sulfur of the bile. There was a distinct but less striking increase when sodium cholate alone was given by mouth. There was no increase in bile sulfur when cystine alone was fed. Wohlgemuth followed the sulfur of the urine in cats before and after administering cystine. There was a large amount of neutral sulfur excreted, and, as there was no cystine in the urine, he concluded that all the cystine had been changed into taurine. This reasoning may be open to certain objections.

Gibson (5) gave three injections of bromobenzol to cats at 8 to 10 hour intervals to deflect the cystine from its usual cycle and found a marked decrease in the alcohol-soluble sulfur of the bile. He does not mention whether the cats were kept upon a uniform diet nor does he state their clinical condition after administering the drug. The cats were sacrificed 5 to 8 hours after the last injection. We know that this decrease in bile sulfur might have been caused by a diet low in meat protein nitrogen, by refusal of food, or by the intoxication from the bromobenzol.

Lifschütz (8) found that cholic acid gives the same color changes and spectrum as cholesterol or oxysterol when treated with acetic acid, benzoylsuperoxide, and sulfuric acid. Flury (3) states that the acids obtained by the oxidation of cholesterol may be placed in the pharmacological group of bile acids and saponins from their pharmacological behavior. He thinks that they may occur as intermediate steps in the formation of bile acids from cholesterol.

Excellent work has been reported by Schrötter, Weitzenböck, and Witt (11, 12). They found that cholesterol, cholic acid, oil of turpentine, and camphor all give the same compound, rhizocholic acid, when treated with concentrated sulfuric acid, mercury, and nitric acid. Rhizocholic acid is:



and was identified carefully in each case. From this fact they conclude that cholic acid and cholesterol belong to the terpene series.

Pregl (10) states that cholic acid is closely related to turpentine and camphor and is a hexahydroxylbenzene. Moersch (9) in 1913 attempted to show the relation of cholic acid to cholesterol by making a trichlor derivative from each. The cholic acid formed a compound with 11 atoms of chlorine, and cholesterol with 10 chlorine atoms.

Hammarsten (7) states that in certain animals (shark) the bile acids are replaced by scymnol sulfuric acid, made up of a substance he calls "scymnol" which is allied to cholic acid or cholesterol paired with sulfuric acid.

D'Amato (1) found that dogs fed on a lipid diet gradually increased the excretion of both cholesterol and bile acids in the bile. The increase noted in bile acids is very small and within the limits of error of the method employed and surely within the physiological variations which are observed so frequently in routine collection periods.

Goodman (6) thought that the broken down red blood corpuscles might be available for the formation of bile constituents; *i.e.*, the cholesterol might be the mother substance of cholic acid. He used a method in which he weighed the cholic acid excreted. The diet consisted of dog biscuit (10 gm. of nitrogen per day), varied with red blood cells, egg white, coagulated horse serum, calves' brains, and cholic acid. The cholic acid caused a marked increase in the cholic acid content of the bile, but the bile cholesterol was unchanged. This cholic acid increase might have been due to cholic acid excreted as such, and not to taurocholic acid, for his method determines any cholic acid dissolved in the bile, whether united with taurine or not. The egg albumin increased the cholic acid but not the cholesterol. He used but one dog, and that dog lived only 4 weeks. For this reason and because of inaccuracies in his methods we do not attach much importance to his results. Because there is such a small amount of cholesterol present in the blood cells, Goodman says that in a human being there would be necessary about 60 per cent blood destruction per day, and even more in the dog, which of course is unthinkable. This theory at best could explain only a small part of the actual cholic acid production. Our experiments give no support to this suggestion.

$$\begin{array}{c}
 \text{CH}_3 \diagup \text{CHCH}_2\text{CH}_2 - \text{C}_{11}\text{H}_{17} \\
 \text{CH}_3 \diagdown \\
 \begin{array}{ccccc}
 & \text{CH} & & \text{CH} & \\
 \text{H}_2\text{C} & & \text{CH} & & \text{CHCH}_2 \\
 | & & | & & | \\
 \text{H}_2\text{C} & & \text{CH}_2 & & \text{CH} \\
 & \diagdown & & \diagup & \\
 & \text{CHOH} & & \text{CH}_2 &
 \end{array}
 \end{array}$$
$$\text{COOH}-\text{CH} \begin{array}{c} \text{CH}_2 \text{ CH}_2 \\ \text{CH}_2 \text{ CH}_2 \end{array} \text{CH}-(\text{CH}_2)_4-\text{CH} \begin{array}{c} \text{CH}_2\text{OH} \quad \text{CH}_2\text{OH} \\ \text{CH} \quad \text{OH} \\ \text{CH} \quad \text{CH}_2 \\ \text{CH} \quad \text{CH} \\ \text{CH}_2 \quad \text{CH} \\ \text{CH} \quad \text{CH} \\ \text{CH} \quad \text{OH} \end{array}$$

Table L presents two similar experiments in which cystine was given intravenously with no positive influence on the bile acid output, but with a decided effect upon the bile unhydrolyzed amino nitrogen fraction. This may indicate an excretion in the bile of cystine or taurine under these conditions. On the following day a similar injection of cystine followed by cholic acid by stomach resulted in a great increase in taurocholic acid excretion and a return of the unhydrolyzed amino nitrogen to normal. This gives evidence that under these conditions the body can change cystine rapidly to taurine, which is then available to combine with the cholic acid. It may be objected that on a mixed diet the feeding of cholic acid will increase the output of taurocholic acid because plenty of taurine is available from the food. Refer to Table XXIV, Paper III, where it is seen that the rise in tauro-

cholic acid does actually reach a higher level after administration of 4.0 gm. of cholic acid with a full mixed diet. It would be desirable to repeat these observations during a fasting period.

Taken together with the experiments of von Bergman and Wohlgemuth we feel reasonably secure in the statement that cystine can produce taurine under physiological conditions, and

TABLE L.
Cystine Intravenously Reacts with Cholic Acid.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	cc.	mg.	mg.	mg.	lbs.	
15-22	Nov. 25	53	0.227	12.04	442	33.3	Mixed diet.
	" 26	28	0.456*	12.75	468	33.0	0.7 gm. of cystine intravenously.
	" 27	51	0.746	38.05	1,395	32.5	0.7 gm. of cystine intravenously + 2.1 gm. of cholic acid by stomach.
17-34	Nov. 25	35	0.198	6.93	254	30.3	Mixed diet.
	" 26	40	0.356*	14.24	522	30.3	0.7 gm. of cystine intravenously. Vomited.
	" 27	76	0.453	34.45	1,264	30.3	0.7 gm. of cystine intravenously + 2.1 gm. of cholic acid by stomach. Vomited a little of cholic acid solution.

* The *unhydrolyzed* bile gave a high *unhydrolyzed* NH_3 showing the presence of an excess of cystine or taurine uncombined with cholic acid. The following day when cholic acid was given this *unhydrolyzed* NH_3 had returned to normal which would indicate that all the cystine had been synthesized into taurocholic acid.

this taurine is available to combine with an excess of cholic acid. This holds good apparently whether the cystine is fed by mouth (von Bergman) or given intravenously (Table L).

In comparing Table LI with Tables XXXI and XXXIII of Paper IV, one can see that these variations are within the normal fluctuations of these same dogs on a sugar diet.

Tables LI and LII are to be considered together and they furnish strong evidence that *cholesterol* alone or fed with taurine exerts no influence upon the excretion of taurocholic acid. This holds for periods of fasting as well as for periods of mixed diet. The amounts of cholesterol administered (3 to 4 gm.) are sufficient to convince any investigator that this substance does not play a part in bile acid metabolism. There is no immediate reaction nor any

TABLE LI.
Cholesterol Feeding—Sugar Diet—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.					
17-151	1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	Diet of 75 gm. of cane sugar, 100 gm. of glucose.
	Average 11 days.			8.05	295		3.45		
	Apr. 6	15	0.402	6.03	220		2.52	35.0	4 gm. of cholesterol.*
	" 7	17	0.556	9.45	347	18.6	2.13	35.1	
	" 8	17	0.416	7.08	260	14.2	2.57	34.4	
18-23	1918	cc.	mg.	mg.	mg.	mg.	gm.	lbs.	Diet of 75 gm. of cane sugar, 75 gm. of glucose.
	Average 11 days.			8.66	318		3.23		
	Apr. 6	37	0.171	6.32	232	14.2	3.30	25.8	4 gm. of cholesterol.*
	" 7	31	0.269	9.34	343	13.0	2.58	25.7	
	" 8	37	0.125	4.62	169	18.7	2.18	25.7	

* Given in gelatin capsule at beginning of 6 hour collection.

delayed effect to be observed. In Table LII the taurine was given to insure an excess of this substance in the body to combine with any amount of cholic acid available. Table LII illustrates the inhibition of bile flow due to sugar by mouth (Dog 15-22). Vomiting on certain occasions may be associated with a decreased flow of bile in these fistula dogs. The amount of ether used to dissolve the cholesterol (8 to 10 cc.) will have no influence upon the bile excretion.

Tables LIII and LIV are similar experiments which show that red blood cells by mouth have no effect upon the excretion of bile acids. The control periods of 5 days and the red blood cell feeding periods give figures which are practically identical. The

TABLE LII.

Taurine Plus Cholesterol Feeding—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	cc.	mg.	mg.	mg.	lbs.	
18-93	Sept. 11	76	0.210	15.95	586	31.5	Mixed diet.
	" 12	58	0.320	18.55	681	32.5	1 gm. of taurine,* 3 gm. of cholesterol. Some diarrhea.
	" 13	76	0.225	17.10	628	34.0	Intoxicated by ether. Hb. 126 per cent. R. B. C. 6,430,000.
15-22	June 6	3.6	0.642	6.40	235	28.2	Average 10 days fasting.
	" 7	10	0.616	6.16	226	27.4	100 gm. of cane sugar and 25 gm. of glucose after 10 days fasting with bile exclusion.
	" 7	10	0.616	6.16	226	27.4	Vomiting. 2 gm. of taurine,* 3 gm. of cholesterol.
18-54	Sept. 3	42	0.170	7.14	262	27.0	Mixed diet.
	" 4	46	0.141	6.49	238	27.4	Hb. 110 per cent. R. B. C. 5,515,000.
	" 5	58	0.113	6.56	241	28.0	1 gm. of taurine* and 3 gm. of cholesterol. Intoxicated by ether.

* Taurine dissolved in water. Cholesterol dissolved in ether, and given by stomach tube at beginning of 6 hour collection.

mixed diet gives considerable daily fluctuation, but the averages are nearly uniform. The addition of 10 gm. of dried red blood cells does not influence the curve of bile acid excretion. This amount of red cells contains an appreciable amount of cholesterol, but of course not comparable to the large amounts used in Tables

LI and LII. We have other experiments with brain feeding which show the same negative influence on bile acid excretion.

Table LV shows the negative reaction following the intravenous injection of laked red blood cells. This again illustrates how easily we may dissociate the excretion curves of bile pigments and

TABLE LIII.

Red Blood Cell Feeding—Bile Acids Unchanged.

Dog 18-23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
July 15	56	0.272	15.23	559	31.8	Mixed diet. Absolute bile exclusion.
" 16	63	0.254	16.00	587	33.3	Hb. 115 per cent. R. B. C. 6,200,000.
" 17	67	0.297	19.90	731	32.5	
" 18	71	0.410	29.10	1,068	32.0	
" 19	62	0.324	20.18	741	32.3	
Average.....			20.00	734		
July 22	53	0.254	13.47	495	31.0	Mixed diet + 10 gm. of R. B. C.*
" 23	60	0.262	15.72	576	32.3	" " + 10 " " "
" 24	57	0.302	17.20	628	32.5	" " + 10 " " "
" 25	64	0.271	17.35	637	32.8	" " + 10 " " "
" 26	62	0.326	20.20	742		" " + 10 " " "
Average.....			16.80	617		

* Prepared by washing red blood cells in normal saline 3 times. The residual cells were dried by warm air and ground to a powder, 10 gm. of red cells made into an indefinite solution emulsion mixture, flavored with a little sugar and salt, made up to 400 cc. with water, and given by stomach tube.

bile acids. The intravenous injection of large amounts of laked red blood cells will cause a prompt and large increase in the output of bile pigments but no increase in bile acid excretion. There is no immediate and no delayed bile acid reaction which we have been able to observe. Some investigators have recorded a drop in bile acid excretion following intravenous injections of hemoglobin but our experiments are clear cut and negative in this respect. One

observes only the normal physiological fluctuations. A possible explanation of the observed depression of bile acid excretion is the febrile reaction which sometimes is observed following hemoglobin injections. No such reaction was observed in these experiments.

It is well to note that the whole laked cells were given—that is, stroma and hemoglobin. After the blood had been laked by dis-

TABLE LIV.

*Red Blood Cell Feeding—Bile Acids Unchanged.
Dog 18-54. Bile Fistula and Splenectomy.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
July 22	37	0.268	9.91	363	28.0	Mixed diet. Bile exclusion.
" 23	41	0.213	8.71	319	28.3	
" 24	18	0.315	5.67	208	27.8	
" 25	33	0.299	9.87	362	27.5	
" 26	45	0.282	12.68	466		
Average			9.40	345		
July 30	65	0.125	8.13	299	28.0	Hb. 100 per cent. R. B. C. 5,075,000.
" 31	48	0.237	11.36	417	28.3	Mixed diet + 10 gm. of R. B. C.*
Aug. 1	50	0.284	14.20	522	27.8	" " + 10 " " "
" 2	37	0.145	5.36	196	27.5	" " + 10 " " "
Average			9.7	356		Tube out. About 10 cc. lost.

* Red blood cells prepared as in Table LIII.

tilled water it was shaken thoroughly to insure a complete injection of stroma and cell fragments. It has been suggested repeatedly that the red cell stroma and perhaps the contained cholesterol were waste products which normally came to the liver to be transformed into bile acids and eliminated in the bile. This is an attractive hypothesis which is delightfully simple, but like many others it has no basis of experimental fact and should be put aside even if with regret.

Since Schrötter, Weitzenböck, and Witt were able to make rhizocholic acid from cholic acid, cholesterol, turpentine, or camphor, we thought it might be possible to produce cholic acid in the animal body by feeding either turpentine or camphor. Taurine was given on the last day in each experiment (Table LVI) so that any cholic acid which might be formed in the organism would unite with the taurine and be excreted in the bile as tauro-

TABLE LV.

Laked Red Cells Intravenously—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	cc.	mg.	mg.	mg.	lbs.	
18-23	July 29	66	0.262	17.28	637	30.5	Mixed diet.
	" 30	72	0.223	16.05	590	32.3	Laked blood.*
	" 31	58	0.349	20.25	744	32.3	Hb. 120 per cent. R. B. C. 6,375,000.
	Aug. 1	63	0.287	18.18	678	31.8	Laked blood.*
	" 2	51	0.413	21.03	772	32.3	
18-93	July 29	68	0.207	14.07	517	30.3	Mixed diet.
	" 30	66	0.241	15.90	584	31.8	Laked blood.*
	" 31	53	0.280	14.85	545	32.3	Hb. 135 per cent. R. B. C. 6,380,000.
	Aug. 1	70	0.229	16.02	588	32.3	Laked blood.*
	" 2	64	0.315	20.15	740	33.3	

* 100 cc. of sterile defibrinated normal blood, centrifuged, red cells washed and laked with water, made up to 100 cc., and kept on ice over night; rendered isotonic, warmed, shaken, and given intravenously by hypodermic needle 2 hours after start of collection.

cholic acid. This was probably an unnecessary precaution when we recall the great output of taurocholic acid which follows a cholic acid feeding during periods of mixed diet. This indicates an abundant source of taurine in the body during periods of liberal feeding, more than enough to combine with any expected excess of cholic acid.

Careful scrutiny of Table LVI reveals no fluctuations other than those observed in control periods, whether terpene hydrate alone or combined with taurine was given by stomach. There is no

TABLE LVI.

Terpene Hydrate With and Without Taurine by Mouth.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurine added in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	cc.	mg.	mg.	mg.	lbs.	
15-22	Sept. 3	22	1.150	25.30	929	33.3	Mixed diet. Hb. 150 per cent. R. B. C. 7,455,000.
	" 4	47	0.214	10.05	369	34.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 5	45	0.381	17.15	630	33.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 6	38	0.388	14.73	541	32.8	
18-23	Aug. 27	57	0.261	14.86	545	30.8	Mixed diet.
	" 28	57	0.343	19.55	718	31.8	0.25 gm. of terpene hydrate.*
	" 29	70	0.309	21.63	794	33.3	0.5 gm. of terpene hydrate.*
	" 30	53	0.434	23.00	844	33.3	0.5 gm. of taurine.
18-54	Sept. 10	61	0.112	6.84	751	28.8	Mixed diet.
	" 11	82	0.238	19.50	716	29.0	
	" 12	81	0.186	15.05	552	28.5	1 gm. of terpene hydrate,* 1 gm. of taurine by stomach tube. Diarrhea.
	" 13	47	0.155	7.29	267	27.8	
17-181	Sept. 10	49	0.292	14.30	525	25.0	Mixed diet.
	" 11	42	0.280	11.75	431	25.3	
	" 12	67	0.267	17.85	655	26.0	1 gm. of taurine, 1 gm. of terpene hydrate.* Diarrhea.
	" 13	42	0.324	13.60	499	25.0	
18-93	Sept. 3	64	0.285	18.25	670	30.5	Mixed diet. Hb. 126 per cent. R. B. C. 6,430,000.
	" 4	44	0.073	3.94	144	33.5	1 gm. of taurine and 1 gm. of terpene hydrate.* Diuresis.
	" 5	54	0.183	9.89	363	32.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 6	64	0.291	18.61	684		

* Terpene hydrate dissolved in a little dilute alcohol; taurine dissolved in water, given by stomach tube at beginning of 6 hour collection.

chologogue action and no change in the bile concentration and total amounts of bile acids.

Table LVII shows the results of administration of camphor by mouth. Repeated doses of spirits of camphor have no effect upon the bile acid excretion. Similar observations have been made on

TABLE LVII.

*Camphor Feeding With and Without Taurine by Mouth.
Dog 15-22. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	cc.	mg.	mg.	mg.	lbs.	
July 29	48	0.290	13.92	511	33.5	Mixed diet.
" 30	29	0.155	4.49	165	34.3	0.2 gm. of camphor* by stomach tube. Hb. 135 per cent. R. B. C. 6,430,000.
" 31	35	0.292	10.22	375	34.3	0.4 gm. of camphor by stomach tube.
Aug. 1	38	0.430	16.34	600	33.5	0.6 gm. of camphor by stomach tube. Considerable salivation.
" 2	14	1.030	14.41	529	33.5	
" 5	47				33.8	0.4 gm. of camphor by stomach tube.
" 6					35.0	0.4 gm. of camphor by stomach tube.
" 7	53	0.269	14.25	523	34.3	0.4 gm. of camphor by stomach tube.
" 8	63	0.222	14.00	514	33.3	0.4 gm. of camphor and 1 gm. taurine by stomach tube.
" 9	40	0.439	17.56	644	34.0	0.4 gm. of camphor by stomach tube.

* Camphor given as a 10 per cent solution—spirits of camphor. Diuresis was uniformly noted.

other dogs. Obviously there is no immediate and no delayed reaction which can be attributed to the action of this drug.

It is evident that neither camphor nor terpene hydrate have any effect on the excretion of taurocholic acid. Cholesterol, turpentine, cholic acid, and camphor may all belong to the same group chemically, but physiologically they certainly are not closely related, at least as regards bile acid metabolism.

SUMMARY.

Taurine as found in the body is derived in all probability from the cystine of the food or body protein. Taurine appears to be present in excess of the amount needed to combine with the cholic acid of normal metabolism.

Cholic acid is the limiting factor which determines the level of bile acid excretion in the bile. The origin and fate of cholic acid have not been satisfactorily determined.

Cholesterol fed alone or combined with taurine causes no change in the excretion of bile acids. This gives no evidence of any physiological relationship between cholesterol and cholic acid.

Red blood cells fed by mouth or hemolyzed and injected intravenously have no influence upon the level of bile acid excretion.

Terpene hydrate and camphor fed alone or combined with taurine do not influence the curve of bile acid excretion.

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DETERMINATION OF AMMONIA IN THE BLOOD.

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(Received for publication, May 1, 1919.)

The estimation of ammonia in the blood is a difficult matter not only because ammonia is present in very minute quantities, but also because it is rapidly split off from some labile components of shed blood. The removal of the ammonia by means of an air current is subject to a source of error in as much as the amount of ammonia recovered may vary with the rapidity of the aeration and the temperature.¹ Besides, the color produced by Nesslerization is too weak to be compared in the colorimeter unless the apparatus is specially adjusted for the purpose,² while the microtitration of Barnett³ requires considerable manipulation which makes the method somewhat cumbersome.

The method which we developed in this laboratory (with the view of future application to the study of some problems) seeks to avoid these disadvantages. In the first place, the blood proteins are precipitated as soon as the blood is taken to prevent the splitting off of ammonia. Secondly, the ammonia in the blood filtrate is absorbed with the aid of permutit⁴ which, of course, does away with the necessity of driving off the ammonia by aeration. Furthermore, by the addition of a measured quantity of ammonia to the filtrate, it is possible to examine the color produced by the Nesslerization without changing the colorimeter.

Before this procedure was adopted for the determination of ammonia in the blood, we performed many tests with solutions of albumin in Ringer's solution to which standard ammonium sulfate

¹ Henriques, V., and Christiansen, E., *Biochem. Z.*, 1917, lxxx, 297.

² Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 532.

³ Barnett, G. D., *J. Biol. Chem.*, 1917, xxix, 459.

⁴ Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

had been added to produce a concentration of ammonia similar to that in normal blood. The albumin was precipitated with a 25 per cent solution of *m*-phosphoric acid. To the water-clear filtrate we then added 0.5 mg. of ammonia and sodium hydroxide nearly to neutralize the excess of acid. The mixture was well shaken with several grams of permutit. This was allowed to settle and, after decanting the supernatant fluid, it was washed twice with ammonia-free distilled water. In the meantime a control consisting of an equal volume of Ringer's solution to which was added the same amount of *m*-phosphoric acid and sodium hydroxide to leave the mixture slightly acid, and also containing 0.5 mg. of ammonia, was treated in a similar manner with permutit. The washed permutit of the control and unknown was transferred to 100 cc. volumetric flasks, the absorbed ammonia set free with 1 cc. of 10 per cent sodium hydroxide, and Nesslerized with 15 cc. of Folin-Nessler's reagent. Both flasks were then filled up to the mark, and the color compared in a Duboscq colorimeter. The difference in intensity of the produced color was due to the ammonia present in the original albumin solution. In all our experiments with the artificial "blood," the ammonia recovered by the above method was invariably in accord with the theoretical expectation. These preliminary experiments convinced us, therefore, that the procedure may be properly applied to the determination of the ammonia in the blood.

All the determinations recorded below were made on human blood. This was drawn from the vein of the arm into a large luer syringe containing a little powdered potassium oxalate, and immediately transferred to a 250 cc. volumetric flask. The flask contained approximately 200 cc. of ammonia-free distilled water, and 10 cc. of *m*-phosphoric acid, and was carefully weighed. It was weighed again as soon as the blood was put into it, and enough 25 per cent *m*-phosphoric acid added to have 1 cc. of acid for every 2 gm. of blood taken. The flask was filled to the mark with ammonia-free water and the contents well mixed. It was then allowed to stand for several hours. Duplicate analyses with sheep's blood showed that the length of time during which the material was allowed to stand had no influence upon the amount of ammonia recovered. Generally we let the flasks stand over night because the blood was obtained late in the

afternoon, but sometimes when a sample was gotten in the morning, the contents were filtered and analyzed within 2 or 3 hours. If the material is filtered sooner the filtrate is usually colored and turbid.

The precipitated material was filtered through a folded paper into a graduated cylinder. Generally 200 cc. of a perfectly clear and practically colorless fluid could be collected within a few minutes. This was transferred to a large Erlenmeyer flask and the excess of *m*-phosphoric acid neutralized with 10 per cent sodium hydroxide (sp. gr. 1.12). We found from experience that it required 1 cc. of the hydroxide for every 4 cc. of acid used in the precipitation to reduce the acidity of the mixture to a degree where it was just barely sufficient to redden litmus paper.

The control was made up with 200 cc. of Ringer's solution to which the same quantity of *m*-phosphoric acid and alkali were added. We found difficulty in matching the colors exactly unless the control was treated in just this manner. 5 cc. of the standard ammonium sulfate solution, equivalent to 0.5 mg. of ammonia, were then added to the filtrate from the blood and to the control, and the ammonia in each absorbed with 10 gm. of permutit. The permutit was carefully weighed and washed four times with ammonia-free water to remove all fine particles. After such preliminary treatment the permutit would settle to the bottom quickly like sand and leave no silt-like suspension in the liquid. The flasks were shaken by a continuous rotary motion for exactly 3 minutes. The supernatant fluid was then poured off and the sediment of permutit washed twice with ammonia-free distilled water. The permutit was transferred quantitatively to a 100 cc. volumetric flask, and Nesslerized in the manner already described.

In liberating the ammonia from the permutit, we found it more desirable to use only 1 cc. of the sodium hydroxide, which experiments have shown us to be sufficient to set the ammonia free quantitatively. With this smaller quantity of sodium hydroxide, we practically never had any trouble from the development of turbidity upon Nesslerization.

The following represents a series of determinations of ammonia in the blood of normal individuals:

No.	Subject.	Age.	Quantity of blood.	NH ₃ per 100 gm. of blood.
		<i>yrs.</i>	<i>gm.</i>	<i>mg.</i>
1	Man.	27	21.8	0.30
2	"	41	22.0	0.27
3	Girl.	19	24.0	0.14
4	Man, colored.	32	25.0	0.16
5	Girl.	21	24.0	0.21
6	Man.	60	22.0	0.18

A NOTE ON THE DETERMINATION OF THE INORGANIC CONSTITUENTS OF BLOOD AND OTHER PHYSIOLOGICAL MATERIAL.

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(Received for publication, May 14, 1919.)

In the determination of the inorganic constituents of physiological material it has been usual to destroy completely the organic constituents either by ashing or by treatment with sulfuric and nitric acids. Each of these has its advantages and its disadvantages. There are some objections that are common to both. The oxidation of large quantities of organic substances is troublesome and frequently involves danger both of loss of the substance to be determined and of too great addition of other substances that may interfere with the determination. Any procedure that would reduce the amount of substance to be oxidized, or make this step unnecessary, would seem to be desirable.

Moreover, certain elements such as phosphorus are present both in organic and inorganic combination. It is desirable to be able to distinguish between them. For this purpose it is advisable to avoid high temperatures, as well as high concentrations of either acid or alkali.

The chief organic constituents of animal material are proteins and fats (including lipins). Coagulation by heat has been extensively used to remove the proteins and, with them, the fats. It is very simple but involves the danger of decomposition of organic compounds of the element in question. Moreover, a certain amount of the protein escapes coagulation and frequently interferes with the subsequent procedure.

The author has used for the determination of phosphates,¹ sulfates, calcium, sodium, and potassium² a method which he

¹ Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369; 1915, xxi, 29; 1916, xxv, 431; *Am. J. Med. Sc.*, 1914, cxlvii, 225; *J. Pharm. Exp. Therap.*, 1915, vii, 57.

² Greenwald, I., *J. Pharm. Exp. Therap.*, 1918, xi, 281.

believes to be of wide applicability. Because it has been described only incidentally in the presentation of the results obtained with it, it does not seem to have been used by others as much as it otherwise might have been.

The method depends upon the precipitation of the protein and fats by means of picric acid. The exact procedure may vary with the nature of the determination. With blood, it is advisable to dilute to almost 10 volumes with water, add 1 per cent of acetic, hydrochloric, or sulfuric acid, saturate with picric acid, and then dilute to an exact multiple. After standing a few minutes, the mixture is ready for filtering and measured portions are taken for the analyses. The liquid is protein-free and may be oxidized in less than one-tenth the time required for whole blood. In many cases it is unnecessary to oxidize at all.

The method has been employed by Chapin and Powick,³ Feigl,⁴ and by Meigs, Blatherwick, and Cary⁵ for the determination of phosphates, by Van Slyke and Donleavy⁶ for the determination of chlorides, and, slightly modified, by Halverson and Bergeim⁷ for the determination of calcium.

The values obtained by the author² for the sodium, potassium, calcium, and "acid-soluble" phosphorus of dog blood and serum are given in Table I.

TABLE I
Inorganic Constituents of Dog Blood and Serum.

	Na	K	Ca	"Acid-soluble" P.
	mg.	mg.	mg.	mg.
100 gm. of blood.....	286	26.7	18.4	16.4
100 cc. " "	280	24.0		
100 " " serum.....	297	16.3	12.0	3.63
100 " " "			19.6	
100 " " "	320	27.3	20.2	3.13
100 " " "	304	15.2	21.6	3.50
100 " " "	330	13.9	14.0	3.64
100 " " "	335	15.8	15.3	2.60

³ Chapin, R. M., and Powick, W. C., *J. Biol. Chem.*, 1915, xx, 97.

⁴ Feigl, J., *Biochem. Z.*, 1917, lxxxi, 380; 1917, lxxxiii, 81, 218; 1917, lxxxiv, 264; 1918, lxxxvi, 395; xcii, 1.

⁵ Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, i.

⁶ Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

⁷ Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.

THE ABSENCE OF FAT-SOLUBLE A VITAMINE IN CERTAIN DUCTLESS GLANDS.*

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Of late a good deal of emphasis has been placed upon the possible value of the fat-soluble A accessory in the treatment of certain diseases such as rickets, pellagra, and xerophthalmia. It is known for example that some of the oils, such as cod liver oil, which are admitted to be of therapeutic value, contain a vitamine or food hormone. In 1909 Stepp (1) concluded from his experimental studies on rats that certain fats or related substances, such as lipins, which are soluble in fat solvents, were essential for maintenance and growth. McCollum and Davis (2) and Osborne and Mendel (3) came to the same conclusion in 1913 when they discovered that butter fat stimulated growth in young rats that had thrived normally for a long time and then had ceased to grow. Osborne and Mendel (4) found that this accessory resided in the butter oil fraction and had no relation to lipoids; that it was soluble in absolute alcohol, and when introduced into the diet cured an eye trouble which later McCollum classed as a form of xerophthalmia (5). McCollum and associates who designated this accessory as fat-soluble A (6) found: that, if it was present in animal tissues, it was removed with the fats when they were dissolved out by the ordinary solvents as ether; but in vegetable tissues (plants, seeds, or leaves), it remained behind when the oils were extracted with ether, benzine, chloroform, or acetone (5, 7). Hot alcohol, however, removed it from the residue (7, 8).

* Read before the Buffalo meeting, American Chemical Society, April 9, 1919.

The following table gives a current summary of the presence or absence of the fat-soluble A vitamine in food substances, based upon published results.

Present.	Absent or very little.
(a) Animal Source:	(a) Animal Source:
Butter fat (2,3).	Lard (2,4).
Egg yolk and fat (2,4,9).	Pig heart (13,14).
Cod liver oil (9).	
Beef oil (10).	(b) Vegetable Source:
Oleomargarine (10,11).	Olive oil (2).
Cod testicles (12).	Almond oil (9).
Pig kidneys (12,13,14).	Oils from maize, linseed,
Pig liver and liver oil (14).	sunflower, soy beans, and
Fat fish and fish oils (15).	wheat (17).
Dried and unsweetened condensed milk (16).	Cotton seed oil (12, 17).
	Nut margarine (11).
(b) Vegetable Source:	Vegetable margarine (11).
Corn, wheat germ, rye, and	White beans (24).
oats (13).	Barley (25).
Leaves of plants (5,7,17,18).	Potato (26).
Cotton seed flour and oil (19).	
Flaxseed, millet, and hemp	
seed (7).	
Soy beans (20).	
Peas (21, 22).	
Bananas (23).	

From the above list, it will be seen that one of the ductless glands, the cod testicles, has been found to contain the fat-soluble A in the ether extract. In this report we add our findings, made upon the fats from three other ductless glands—pancreas, thymus, and suprarenal.

The glands were ground and extracted with either acetone or benzine. The fats were redissolved in ether and again filtered. They were then incorporated in a diet that had been found to be adequate for growth except for a lack of the fat-soluble A accessory. The deficient diet was first fed to rats in order to bring them down to a plane where they would show definite symptoms of a lack of this vitamine such as loss in weight, poor physical condition, and xerophthalmia. The rats were then fed the modified diet and the results compared with control tests (Chart 1) carried out under the same conditions. 10 per cent of the glandular fat was used.

The weight curves, given in Charts 2, 3, 4, and 5, show definitely that benzine or acetone did not extract from the pancreas, thymus, and suprarenal glands a fat that contained the fat-soluble A vitamine. Therefore, in the use of desiccated products of these glands, it is safe to state that any of the ether-soluble fat which is still present (the major portion being removed before desiccation) does not have any therapeutic value from the standpoint of the fat-soluble A vitamine.

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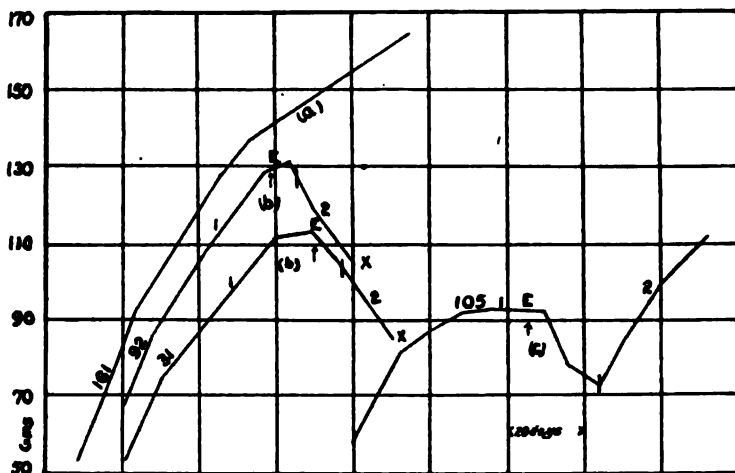


CHART 1. Butter fat. The curves in this chart represent the control groups; (a) rats on a diet with fat-soluble A present, (b) rats on a diet with fat-soluble A absent, and (c) rats that were first put on a deficient fat-soluble A diet, Period 1, and then cured by introducing this accessory into the food mixture, Period 2. "E" designates when xerophthalmia became evident. The basal diet was made up of lactalbumin protein 10 per cent; lard 28 per cent; protein-free milk (the carrier of the water-soluble B vitamins, mineral salts, and lactose) 28 per cent; and starch. 10 per cent of purified butter fat was employed to supply the fat-soluble A and replaced an equivalent amount of lard.

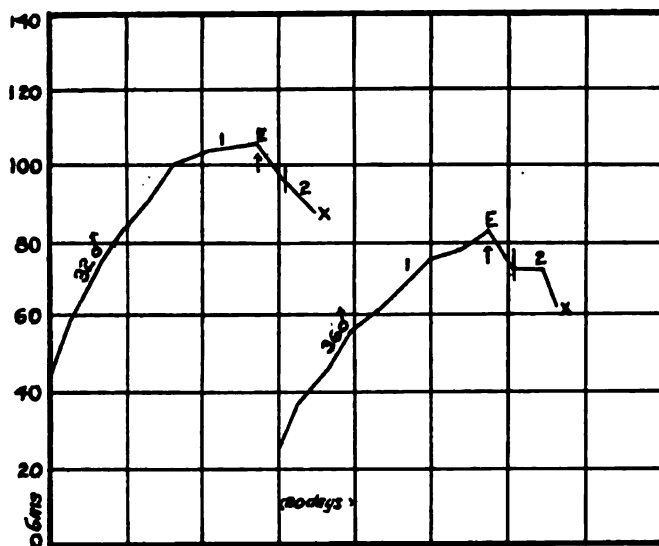


CHART 2. Pancreas fat. In Period 1, the rats were fed the basal diet. Period 2, when the acetone-extracted pancreas fat was used, indicates that the rats did not respond to the change in diet but died as designated by "X."

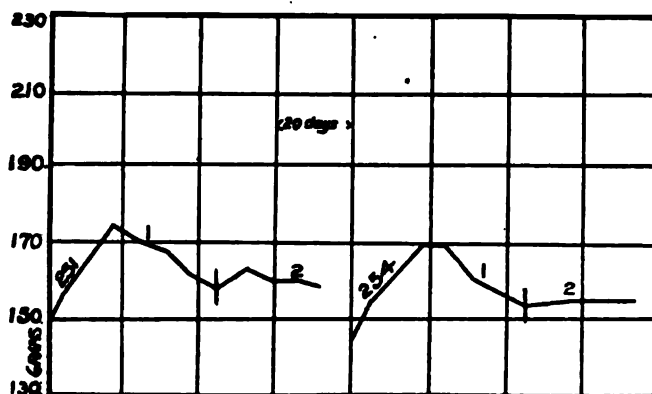


CHART 3. Pancreas fat. In Period 1, the basal diet was used. In Period 2, 10 per cent of the pancreas fat was introduced into the ration. It was obtained by extracting the glands with ether instead of acetone as in Chart 2. These rats were much heavier at the start than those represented in Chart 2. There was a cessation in the loss of weight but no gain in weight and no improvement in bodily condition. The rats finally became so weak that they were killed.

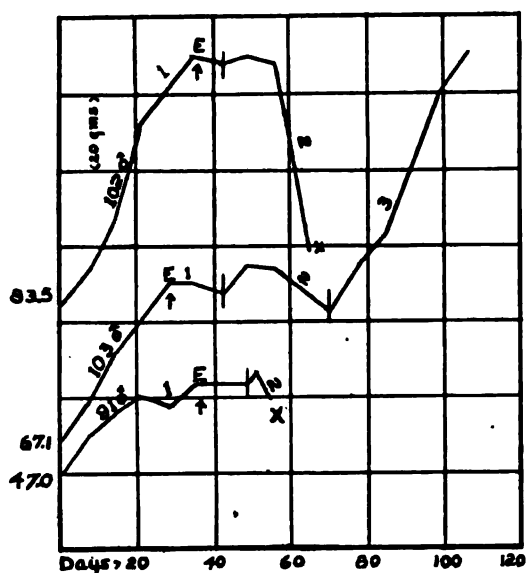


CHART 4. Thymus fat. In Period 1, the diet was the same as the basal food fed the rats represented in Charts 1, 2, and 3. Period 2 shows when the acetone-extracted thymus fat was introduced, there was no beneficial effect resulting, only a continued decline. In Period 3, the equivalent amount of butter fat, substituted for the thymus fat, brought about immediate response.

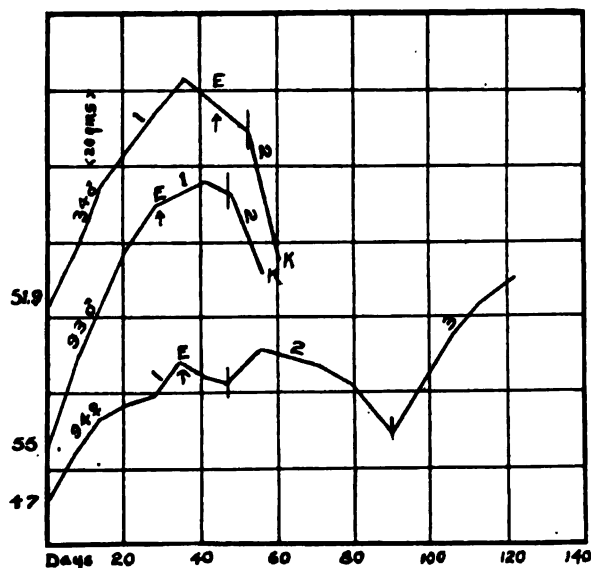


CHART 5. Suprarenal fat. At the end of Period 1, during which the basal diet was fed, 10 per cent of the ether-extracted suprarenal fat was put in the food mixture, in place of so much lard. No growth, gain in weight, or improvement in body took place. Upon introducing 10 per cent of purified butter fat for the suprarenal fat, an immediate response in condition and weight was manifested. Rats 34 and 93 were in such an emaciated condition that they were killed, as shown in the chart by "K."

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A METHOD FOR DETERMINATION OF MINUTE AMOUNTS OF LEAD IN URINE, FECES, AND TISSUES.

By W. DENIS AND A. S. MINOT.

(From the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, May 24, 1919.)

There is great need for a relatively simple and rapid method for the quantitative determination of lead in biological material. At present such a method is not available; indeed we have found that the great majority of the processes described in the literature for even the qualitative detection of lead in urine and feces is extremely laborious and unsatisfactory. The method described below is the result of a critical study of the subject which has extended over a period of five years, and in our hands has given excellent results.

Stated briefly, the process consists essentially in the removal of most of the organic matter by fusion with sodium nitrate, the precipitation of the lead as sulfide, its electrolytic deposition as lead peroxide, and the titration of the iodine liberated from potassium iodide when the lead peroxide deposit is treated with dilute acid. The detailed description of the procedure is as follows.

Determination of Lead in Urine.

Evaporate to dryness 2,000 cc. of urine and allow the dry residue to char slightly. When cool mix the residue with 8 gm. of powdered sodium nitrate and transfer to a small silica evaporating dish or crucible. Heat cautiously until the first violence of the reaction is over, and then more strongly, with a Meker burner or in a muffle furnace, until most of the organic matter has been removed. When cool the material in the dish is transferred to a 300 cc. beaker and treated with 10 per cent hydrochloric acid until a faintly acid reaction is obtained. The acid solution is

then heated to boiling, filtered when still very hot, and the residue on the filter washed thoroughly with boiling water. To the filtrate concentrated ammonium hydroxide is added until a faintly alkaline reaction is obtained, and after the addition of two or three drops of a 1.0 per cent solution of copper sulfate the lead and copper are precipitated by means of ammonium sulfide or hydrogen sulfide.

The suspension of sulfides is transferred to a 50 cc. centrifuge tube, and after centrifuging, the supernatant liquid is poured off, and the sediment washed once with water, two to six times with 0.1 *N* hydrochloric acid, and finally once again with water. The use of the centrifuge for the removal of the precipitated phosphates greatly facilitates the work, when only one or two determinations are being carried on at one time, but when a large number of analyses are being done simultaneously it is generally more convenient to conduct the process by means of decantations from flasks, and to use the centrifuge only when practically all the phosphate precipitate has been removed.

The purpose of this rather elaborate scheme of washing is to remove magnesium, phosphates, and chlorides, all of which interfere with the deposition of lead as peroxide. The use of an acid of a concentration greater than 0.1 *N* appears to give low results. The final residue which consists of the sulfides of lead and copper is transferred to a platinum crucible of 25 cc. capacity by means of 3 cc. of dilute nitric acid (20 parts concentrated nitric acid, sp. gr. 1.42, and 80 parts water) and the tube washed with 3 cc. of distilled water, which are added to the contents of the crucible. The solution is then electrolyzed with a current of 3 to 3.5 amperes and 5 to 6 volts. The crucible serves as the anode, the cathode consisting of a piece of platinum wire 60 mm. long and 1.0 mm. in diameter which is twisted to form a spiral. In our work we have used a storage battery current having an E.M.F. of 16 volts; in addition to the rheostat, an ammeter and voltmeter are kept in the circuit during the entire period of electrolysis. On account of the small volume of the electrolyte, the temperature rises rapidly and generally reaches a maximum of 75°C. within 2 or 3 minutes after the current is turned on. Within 15 minutes all the lead is deposited on the crucible anode in the form of a brown film of lead peroxide, while the copper and

any traces of iron which may be present have been deposited on the cathode.

If the current is turned off while the lead peroxide is in contact with nitric acid a considerable portion of the deposit is dissolved before it is possible to pour off the acid electrolyte. It is therefore necessary to remove the acid liquid by means of a siphon while the electrolysis is still going on. As the acid is drained off, distilled water is introduced and this process is continued until the marker of the ammeter has dropped to zero, showing the removal of practically all electrolytes.

The crucible is disconnected, washed out once or twice with distilled water, and to it are then added 5 cc. of a 5 per cent solution of potassium iodide (free from iodate) and 1 cc. of 25 per cent acetic acid. The lead peroxide is almost instantly decomposed, giving a deposit of yellow lead iodide on the crucible, and a solution of free iodine. The mixture should be allowed to stand for 5 minutes to allow the reaction to be quantitatively completed, and the liquid is then transferred to a small beaker and the iodine titrated with 0.005 N sodium thiosulfate solution using starch as indicator. 1 cc. of 0.005 N sodium thiosulfate solution is equivalent to 0.517 mg. of lead.

This titration is an adaptation of a method in use for many years in technical laboratories for the assay of hydrogen peroxide preparations.¹

Determination of Lead in Feces and Tissues.

About 500 gm. of soft feces or half that amount of more solid material, or 100 gm. of muscle, liver, or other tissue are spread in a thin layer on an ordinary china dinner plate, and the moisture rapidly removed by allowing the plate to stand on a gas or electric hot plate. If the layers are made sufficiently thin the process is usually completed within one or at most two hours.

The dry material is then ground to a coarse powder in a porcelain mortar, mixed with one-half its weight of sodium nitrate, transferred to a silica evaporating dish or crucible, and heated

¹ 0.005 N sodium thiosulfate does not keep well and a fresh preparation should be prepared (by dilution of the 0.1 N solution) at intervals of about 10 days.

with a Bunsen burner. Oxidation begins promptly and must, if too violent, be controlled by removing the burner. After the first reaction is over the dish should be strongly heated for 10 to 20 minutes either with a Meker burner or in a muffle furnace. The subsequent treatment is the same as already described for urine. In the case of feces from patients who have received magnesium sulfate, the precipitate obtained when the acid extract of the fusion is made alkaline is extremely heavy and requires, in the more extreme cases, a more prolonged treatment with dilute hydrochloric acid than is described for urine.

Where quantitative results are not desired the above process may be used for the qualitative detection of lead, as the lead peroxide deposit and the film of lead iodide are of characteristic appearance. As little as 0.1 mg. of lead can easily be detected by this means.

When carried out on the quantitative basis we have been able to recover lead added to urine, feces, and tissues in amounts varying from 0.5 to 4 mg. to the average extent of 95 per cent of the amount added. It is of course obvious that only sodium nitrate and nitric acid which have been tested and found free from lead should be used.

When nitric acid is electrolyzed in a platinum crucible and acidified potassium iodide introduced into the crucible (after the latter has been carefully washed with distilled water) a red color is produced, which gives a blue color with iodine, and which is discharged by one or two drops of 0.005 N thiosulfate solution. We have been unable to find out the cause of this phenomenon. Nitric acid twice distilled from a Jena glass retort gives results essentially similar to the ordinary high grade c.p. nitric acid. On account of this coloration qualitative tests for lead made by this method should not be reported "positive" unless the deposit of lead iodide is present. In quantitative work this "blank" should be determined and the amount of thiosulfate used to discharge the color so obtained should be subtracted from the result of the titration.

THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF COW'S MILK.

By W. DENIS AND A. S. MINOT.

(From the Chemical Laboratory of the Massachusetts General Hospital,
Boston.)

(Received for publication, May 24, 1919.)

But little reliable data is at present available regarding the quantitative relationships of the non-protein nitrogenous constituents of milk.

The qualitative composition of this fraction has been known, to a certain extent at least, for many years. Amino-acids, urea, creatine, creatinine, purine bodies, and ammonia have long been recognized as constituents of this fluid, but on account of the lack of appropriate analytical methods we are still relatively ignorant of the amounts of these bodies present in milk either under normal or under pathological conditions. In a recent paper from this laboratory¹ a description was given of the analytical technique which had been worked out for the quantitative determination of some of the non-protein constituents of milk, and in the present communication we wish to present results obtained by means of these methods on a considerable number of samples of cow's milk.

The results presented in Table I were obtained on the mixed product of four large milk distributors in Boston, and on thirty-eight samples of milk from individual cows which were personally collected by us in three dairies.

An inspection of these results would seem to show that while different samples of mixed milk of a large number of cows give practically uniform results, irrespective of variety of origin, the milk taken from individual cows in different dairies tends to give striking differences in the values for non-protein nitrogen and urea.

¹ Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 353.

TABLE I.

Non-Protein Nitrogenous Constituents of Cow's Milk.

Cow No.	Amount per 100 cc. of milk.						Remarks.
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.*	Preformed creatinine.*	Creatine.*	
	mg.	mg.	mg.	mg.	mg.	mg.	
A	21.9	10.0	4.03	1.4	1.3	2.3	Mixed milk from milk dealer A.
B	21.0	9.0	4.19	1.6	1.4	2.4	" " " " " B.
C	20.0	9.0	4.50	1.6	1.5	2.4	" " " " " C.
D	19.0	6.8	4.18	1.8	1.0	2.2	" " " " " E.
102	22.0	8.0	5.1	1.6	1.1	2.2	Half Holstein, half Jersey, 2 yrs. old; calf 4 months old.
103	25.0	11.0	3.5	1.4	1.0	2.5	Jersey, 4 yrs. old; calf 6 months old.
104	24.0	6.9	3.2	1.4	1.0	2.5	Jersey, 5 yrs. old; calf 8 months old.
105	25.0	8.4	3.2	1.4	1.1	2.3	Jersey, 2 yrs. old; calf 2 months old.
106	25.0	5.2	3.5	1.5	1.0	2.2	Half Jersey, half Guernsey, 6 yrs. old; calf 6 months old.
107	23.0	8.2	3.2	1.6	1.1	2.2	Jersey, 2 yrs. old; calf 6 months old.
108	22.0	5.4	2.6	2.0	1.1	2.0	Jersey, 6 yrs. old; calf 4 months old.
109	23.0	8.6	4.4	1.6	1.0	2.2	Mixed Jersey and Holstein, 2 yrs. old; calf 4 months old.
110	25.0	7.8	3.6	1.4	1.0	2.5	Mixed Jersey and Holstein, 7 yrs. old; calf 3 months old.
111	—	6.0	—	—	1.1	2.3	Mixed breed, 12 yrs. old, about dry; calf 10 months old.
112	22.0	5.7	4.8	1.3	1.2	2.1	Jersey, 7 yrs. old; calf 3 days old.
116	38.0	20.0	4.9	1.6	1.1	2.2	Holstein, 2 yrs. old; calf 3 months old.
117	32.0	15.0	7.3	1.4	1.1	2.3	Holstein, 3 yrs. old; calf 10 months old.
118	34.0	17.4	5.3	1.8	1.2	2.2	Holstein, 10 yrs. old; calf 2 months old.
119	29.0	15.0	4.7	1.6	1.2	2.3	Holstein, 10 yrs. old; calf 8 months old.
44	28.0	7.8	—	—	1.1	2.3	Holstein.
60	30.2	8.3	—	—	1.1	2.2	"
38	25.7	8.0	—	—	1.2	2.0	Mixed breed.

TABLE I—*Concluded.*

Cow No.	Amount per 100 cc. of milk.						Remarks.
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.*	Preformed creatinine.*	Creatine.*	
	mg.	mg.	mg.	mg.	mg.	mg.	
34	24.7	10.8	—	—	1.0	2.5	Holstein.
5	27.5	10.0	—	—	1.1	2.2	"
11	25.0	8.3	—	—	1.1	2.3	Mixed breed.
45	27.0	8.7	—	—	1.2	2.2	" "
2	26.9	7.5	—	—	1.2	2.4	Holstein.
47	30.2	8.0	—	—	1.0	2.6	Jersey.
31	29.2	9.0	—	—	1.0	2.4	Mixed breed.
43	28.0	10.8	—	—	1.0	2.5	Holstein.
42	24.5	6.0	—	—	1.0	2.2	"
37	24.7	8.7	—	—	1.0	2.2	"
41	30.2	8.7	—	—	1.1	2.2	"
58	30.0	9.3	—	—	1.2	2.3	"
28	29.0	8.4	—	—	1.2	2.3	Jersey.
59	24.7	10.0	—	—	1.1	2.5	"
57	27.0	8.0	—	—	1.1	2.4	Holstein.
22	26.0	8.8	—	—	1.1	2.3	Jersey.

* The figures for uric acid, creatinine, and creatine indicate the amounts of these substances, not of their nitrogen.

Thus in Samples 102 to 112 inclusive which were obtained from Dairy M. at Bath, New Hampshire, the values for non-protein nitrogen vary from 22 to 25 mg. and the urea nitrogen from 5.2 to 11 mg. per 100 cc. of milk; Samples 116 to 119 inclusive were obtained from cows belonging to the herd of Dairy B., also at Bath, New Hampshire, and here it will be noted that the figures for non-protein nitrogen and urea nitrogen are on a considerably higher level than in the samples obtained from Dairy M. Samples 5 to 60 were obtained from the dairy of the McLean Hospital at Waverley, Massachusetts, and show values intermediate between those given by the cows in the other two herds.

The above results would seem to point to the influence of food intake, more particularly protein intake, on the relative composition of the non-protein fraction of milk.

Through the courtesy of the owners of the two New Hampshire dairies we were enabled to obtain direct experimental evidence

on this point. Three cows were maintained for us during a period of nearly 2 months on weighed rations. The first samples of milk were taken when the cows were on the ration used for routine feeding by their respective owners. They were then placed on a ration differing in a marked degree as regards protein content from that which had been previously fed, the second milk sample being taken after the cows had eaten this food for a period of 30 days.

The results of this experiment are given in Table II, while in Table III are presented the results of a few observations made on seven cows also fed on rations of known composition.

TABLE II.

*Effect of Protein Intake on the Concentration of Some of the Non-Protein Constituents of Milk.**

Cow.	Date.	Weight of cow. kg.	Nutritive ratio of food	Digestible protein per day. kg.	Non-protein constituents per 100 cc. of milk.						Remarks.
					Total non-pro- tein nitrogen. mg.	Urea nitrogen. mg.	Amino nitrogen. mg.	Uric acid. mg.	Preformed cre- atine. mg.	Creatine. mg.	
M	Dec. 29, 1918	300	1: 12	0.52	22	—	2.6	2.0	1.1	2.0	Jersey.
M	Feb. 24, 1919	300	1: 5.8	0.96	34	19.0	3.1	2.0	1.3	2.1	
B1	Dec. 29, 1918	636	1: 5.2	1.12	32	14.8	7.4	1.4	1.1	2.3	Holstein.
B1	Feb. 24, 1919	636	1: 8	0.80	22	9.8	7.0	1.4	1.2	2.0	
B2	Dec. 29, 1918	545	1: 5.2	1.12	29	15.0	4.8	1.6	1.2	2.1	"
B2	Feb. 24, 1919	545	1: 80	0.80	22	8.6	3.3	1.9	1.2	2.0	

* The foods composing the rations of these cows consisted of hay, oats, barley, cottonseed meal, and silage. The amount of digestible protein, the nutritive ratios, and the ratio of digestible protein to other foods (fats and carbohydrates) of the rations were calculated from the tables given by W. A. Henry and F. B. Morrison (Feeds and feedings, Madison, 16th edition, 1916).

The results presented in Tables II and III leave no doubt of the influence of protein intake on the content of non-protein nitrogen, urea, and amino nitrogen in cow's milk. The other non-protein nitrogenous bodies which have been determined are apparently unaffected by the nature of the food.

TABLE III.

Non-Protein Constituents of Milk of Cows on Weighed Rations.

Cow No.	Weight of cow.	Nutritive ratio of food.	Digestible protein per day.	Non-protein constituents per 100 cc. of milk.						Remarks.
				Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.	Preformed creatinine.	Creatine.	
	kg.		kg.	mg.	mg.	mg.	mg.	mg.	mg.	
120318	1: 7.0	—	28	16.4	3.68	2.5	1.1	2.3		Jersey.
122560	1: 4.7	1.61	27	10.5	4.64	1.9	1.2	2.2		Holstein. Calf 4 months old.
121545	1: 4.7	1.61	25	13.1	3.34	1.9	1.3	2.4		"
123560	1: 8.1	1.00	25	9.8	3.46	2.6	1.1	2.2		"
127—	1: 7.6	0.96	25	10.5	3.30	1.7	1.2	2.2		" Calf 4 months old.
124550	1: 8.1	1.00	19	8.2	3.45	1.8	1.2	2.1		" " 7 " "
126—	1: 10.9	0.66	18	6.8	2.6	1.6	1.2	2.0		" " 7 " "

In Table IV are shown results obtained on a single cow whose milk we were able to examine at more or less regular intervals for a period of several weeks after delivery. This experiment was carried on during the winter months (February and March) and the analyses were started within 3 or 4 hours after the milk had been drawn. It was not possible to arrange to have this animal fed on a weighed ration, so that we are not able to make any definite statement on this point, except to say that with the exception of the first few days after delivery the food remained the same during the remainder of the time that we were making examinations of the milk, and that it was rather high in protein. The validity of the extremely high values for non-protein nitrogen and amino nitrogen noted during the first few days after calving might well be questioned as due to incomplete precipitation of the proteins of colostrum by the methods employed. It should be pointed out, however, that the values for urea nitrogen (which is determined on the entire milk without the necessity of precipitating the proteins) are also extremely high. In the case of another cow from which we were able to obtain samples of milk at more or less regular intervals for a period of 4 days after delivery results were obtained substantially similar to those given in Table IV.

The data collected on this point are difficult to explain. A temporary impairment of renal function during the later stages

of pregnancy, the passage into the blood stream of nitrogenous material formed during the involution of the uterus, or perhaps a combination of both factors might be involved. Studies on human milk, the results of which will be published shortly, lead us to believe that the concentration of many of the non-protein nitrogenous constituents of milk are on the same level as in the maternal blood; it would, therefore, appear to be of considerable interest to repeat the above observations, and at the same time make a series of analyses of the blood. As the experimental material is not available we have been unable to follow out the suggestion.

TABLE IV.

Changes in Content of the Non-Protein Constituents of Cow's Milk during the First Four Weeks after Calving.

Time after calving.	Amount per 100 cc. of milk.					
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Uric acid.	Preformed creatinine.	Creatine.
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
2	74	28.6	7.8	1.5	—	—
14	60	28.0	8.0	1.7	1.3	2.7
26	56	28.0	7.7	1.8	1.3	2.5
36	48	27.0	7.7	1.8	1.2	2.4
50	48	27.0	7.7	1.6	1.2	2.3
74	48	26.6	7.1	1.8	1.2	2.0
98	48	26.0	5.1	1.7	1.3	2.0
122	44	20.0	5.4	1.8	1.3	2.0
146	44	20.6	5.1	1.6	1.2	2.0
432	34	16.6	4.4	1.8	1.3	2.0
668	34	16.0	3.6	1.6	1.3	2.0

SUMMARY.

Figures are given for the non-protein nitrogen, amino nitrogen, urea, uric acid, creatinine, and creatine in cow's milk. It has been shown that the content of non-protein nitrogen, amino nitrogen, and urea is influenced by the character of the food, being increased in high protein feeding and decreased when rations of low protein content are given. The non-protein nitrogen, amino nitrogen, and urea content of colostrum is high and approaches normal values only on the fourth day after delivery.

A REVISED COLORIMETRIC METHOD FOR DETERMINATION OF URIC ACID IN URINE.

BY OTTO FOLIN AND HSIEN WU.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, May 31, 1919.)

For several years acid silver lactate has been used in this laboratory; instead of ammoniacal silver solutions, for the precipitation of uric acid, but publication of the method has never seemed worth while so long as the most cumbersome feature of the uric acid determination, namely the preparation of a permanent standard uric acid solution, had not been solved. A standard uric acid solution of practically unlimited keeping quality was recently described by us¹ in connection with the determination of uric acid in blood. The adaptation of the colorimetric method for uric acid described in this paper has been tested out by ourselves and has also proved thoroughly satisfactory in the hands of our medical students.

Solutions Required.

1. Standard uric acid solution. Before starting to prepare the uric solution a 20 per cent filtered solution of sodium sulfite should be available. Dissolve 1 gm. of uric acid in 125 to 150 cc. of 0.4 per cent lithium carbonate solution and dilute to a volume of 500 cc. Transfer 50 cc. corresponding to 100 mg. of uric acid to each of a series of volumetric liter flasks. Add about 300 cc. of water and then add 500 cc. of clear 20 per cent sodium sulfite solution, mix, dilute to volume, and mix thoroughly. Fill a series of 200 cc. bottles, and stopper very tightly. The reason why a series of small bottles is used as containers is, of course, to reduce the absorption of oxygen from the air.

2. A 10 per cent sodium sulfite solution, kept like the uric acid solution, in small tightly stoppered bottles.

¹ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 102.

3. A 5 per cent sodium cyanide solution.

4. A solution containing 5 per cent of silver lactate and 5 per cent of lactic acid.

5. The uric acid reagent of Folin and Denis. Boil 100 gm. of sodium tungstate with 80 cc. of phosphoric acid (85 per cent) and 700 cc. of water for not less than 2 hours and dilute to 1 liter.

Transfer from 1 to 3 cc. of urine to a 15 cc. centrifuge tube and mix with enough water to make a volume of about 6 cc. Add 5 cc. of the acid silver lactate solution and stir with a very fine glass rod (diameter 1 to 2 mm.). Rinse off the rod with a few drops of water and centrifuge. If enough silver solution has been added the precipitate settles very quickly. Add a drop of silver lactate solution so as to be sure that an excess is present; if a precipitate is formed add more (2 cc.) of the silver lactate solution and centrifuge again. In point of fact the first 5 cc. addition of silver lactate is almost always enough, but it is not safe to omit the test. Pour off the clear supernatant liquid as completely as possible.

To the precipitate in the centrifuge tube add, from a burette, 4 cc. of 5 per cent sodium cyanide solution and stir until a perfectly clear solution is obtained. Pour the contents into a 100 cc. volumetric flask and rinse tube and stirring rod, using for this purpose about 15 to 25 cc. of water. Add 5 cc. of 10 per cent sodium sulfite solution (to balance the sulfite in the standard uric acid solution). Dilute to a volume of about 50 cc.

Transfer to another 100 cc. flask 5 cc. of the standard uric acid sulfite solution, containing 0.5 mg. of uric acid; add 4 cc. of the cyanide solution and dilute to about 50 cc. Then add 20 cc. of saturated sodium carbonate solution to each flask, mix, and finally add *with shaking* 2 cc. of the uric acid reagent. Let stand for 3 to 5 minutes, fill to the mark, mix, and make the color comparison in the usual manner, never omitting to first read the standard against itself. Artificial light (with "daylite" glass) is better than day light for this color comparison.

With the standard set at 20 mm., 10 divided by the reading of the unknown (in mm.) gives the amount of uric acid (in mg.) in the volume of urine taken.

The discarded blue solution should be poured as directly as possible into the drain pipes of sinks on account of the cyanide.

A SIMPLIFIED MACRO-KJELDAHL METHOD FOR URINE.

BY OTTO FOLIN AND L. E. WRIGHT.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, May 31, 1919.)

The purpose of this communication is to describe a macro-Kjeldahl method for the determination of nitrogen in urine which requires very little equipment and by which a urinary nitrogen determination can easily be finished in 20 to 25 minutes.

The hydrolyzing-oxidizing reagent is the same phosphoric-sulfuric acid mixture that is used in the micro method. To 50 cc. of 5 to 6 per cent copper sulfate solution add 300 cc. of 85 per cent phosphoric acid and 100 cc. of concentrated sulfuric acid. 5 cc. of this mixture are used for the destructive digestion of 5 cc. of undiluted urine. 10 per cent solution of ferric chloride is also required. The ferric chloride can scarcely be considered indispensable; but it hastens the digestion, and the iron hydroxide promotes even boiling during the subsequent distillation.

Transfer 5 cc. of undiluted urine to a 300 cc. Kjeldahl flask (Pyrex). Add 5 cc. of the phosphoric-sulfuric acid mixture, also 2 cc. of 10 per cent ferric chloride solution and 4 to 6 small pebbles (to prevent bumping). Boil in a hood over a microburner.¹

Microburners are by far the best to use as a source of heat, because even inexperienced workers or students can not go far astray with them; whereas, large Bunsen burners with their great variations in heating power are much more difficult to regulate. The microburner should give a good strong flame and the top of the burner should be not more than 1 cm. away from the bottom of the flask.

Boil vigorously. In 3 to 4 minutes the foam which forms at first will entirely disappear and the flask becomes filled with dense

¹ A very convenient clamp for holding the Kjeldahl flasks in position is the one listed as No. 24598 in Arthur H. Thomas Company's catalogue. The microburner which we use is listed under No. 1506 in Eimer and Amend's catalogue.

white fumes. When this stage is reached (but no earlier) cover the mouth of the flask with a small watch-glass and continue the vigorous heating for 2 minutes. At the end of 2 minutes dilute urines will already be green or blue and concentrated urines will be a light straw yellow, the black carbonaceous matter being completely destroyed. The flame should then be turned very low and the gentle boiling process should be continued for 2 minutes, making a total boiling period of 4 minutes, counting from the time the watch-glass was put in place. Remove the flame and let the flask cool for 4 to 5 minutes. At the end of 4, or not more than 5 minutes, add first 50 cc. of water, then 15 cc. of saturated sodium hydroxide (50 to 55 per cent), and connect the Kjeldahl flask promptly by means of a rubber stopper and ordinary glass tubing with a receiver containing from 35 to 75 cc. of 0.1 N acid together with water enough to make a total volume of 150 cc., and a drop or two of alizarin red. Florence flasks, capacity 300 cc., of Pyrex glass make excellent receivers for this distillation. As soon as the connection is made with the receiver apply the flame again at full force, but not directly under the center until the acid and alkali in the flask have had time to mix. The contents in the flask begin to boil almost at once and 4 to 5 minutes boiling transfers the whole of the ammonia to the receiver. The contents in the receiver become heated of course, since no condenser is used, but under the conditions described the temperature reached is only 65–70°C.

The only precaution needed in connection with the titration of the distillate (without previous cooling) is that a faint red color shall be accepted as the end-point. The color will deepen on cooling, and when time permits it is more satisfactory to cool in running water before titrating.

While the operator is directed to make prompt connections with the receiver after the alkali has been added to the digestion mixture there is in point of fact very little danger of losing ammonia vapors by being unduly slow in closing the mouth of the flask. Similarly when the receiver contains too little acid and turns pink there is no need for extraordinary haste in adding more acid. The water, though warm, will hold considerable free ammonia.

It will be noted that the digestion period, 30 to 40 minutes of ordinary Kjeldahl determinations, has here been cut down to 4

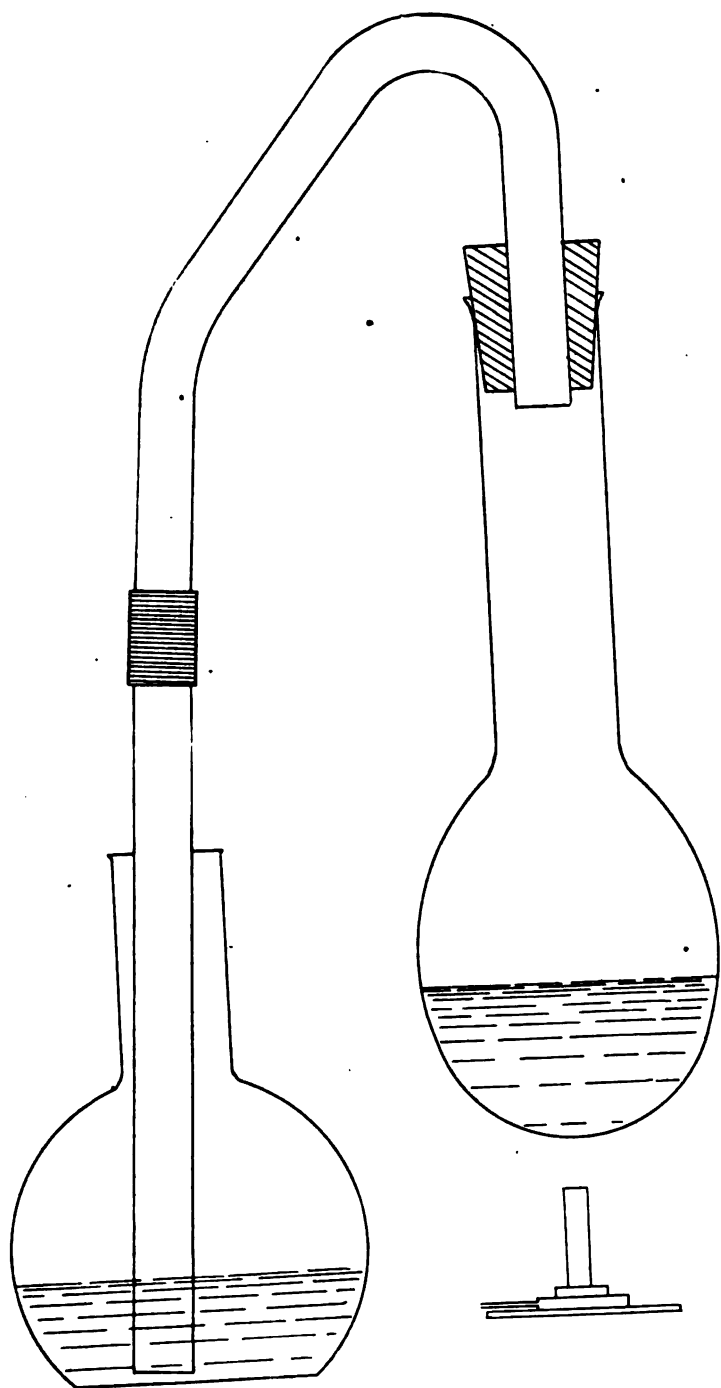


FIG. 1.

minutes and that the distillation period, 25 to 40 minutes, has been cut down to 5, and the only source of heat is an ordinary micro-burner. The delivery tubes are made from glass tubing, small enough to pass into the ready-made holes in rubber stoppers. For the sake of flexibility the delivery tube must consist of two parts connected with a short piece of rubber tubing. (see Fig. 1). It may also be observed that the Kjeldahl flask need not be moved from the time the digestion is begun until the determination is finished, though this involves the continuous use of the hood. Where hood space is scarce the distillation can, of course, be conducted just as well at the desk.

One very satisfactory feature of this new process is that the preliminary steaming out of the condenser for an occasional nitrogen determination is replaced by a simple rinsing of the glass tube.

The simplified distillation process described above can also be used in other determinations involving the removal of ammonia; as, for example, in urea determinations. The elaborate metal condensers now found in every well equipped laboratory are practically superfluous.

The method described in this paper, as far as the destructive digestion is concerned, is primarily intended for urine only. It is not applicable to highly resistant materials, as for example milk, which cannot be completely destroyed within 6 minutes. Urines containing much sugar belong in this class. If fuming sulfuric acid be substituted for ordinary sulfuric acid in the preparation of the hydrolyzing reagent, or if 2 cc. of fuming sulfuric acid are used in addition to 5 cc. of the regular reagent, sugar urines are readily destroyed within the required heating period of 4 to 5 minutes.

THE VITAMINE REQUIREMENT OF YEAST.

A SIMPLE BIOLOGICAL TEST FOR VITAMINE.*

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PLATE 6.

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INTRODUCTION.

Evidence is here presented, based upon direct microscopic observation of the growth of individual yeast cells, that the water-soluble-, beri-beri-preventing vitamine, relatively so abundant in yeast, is necessary for the nutrition of yeast cells themselves.

Pasteur in his famous researches found that yeast would grow in a solution of cane sugar, ammonium salt, and the salts of yeast ash. Since his day it has been generally believed that yeast can grow on such a synthetic medium and that an addition of broken down protein improves such a culture medium for yeast. Pasteur, however, observed the fact that a particle of yeast the size of a pin head (containing several million cells) must be used to inoculate such a solution in order to get appreciable growth and fermentation. This observation we shall see is a very important one.

Wildiers¹ made this same observation in 1901. He also observed that, by adding a little sterile water extract of yeast to a medium containing ammonium salt as its only source of nitrogen, a very small amount of yeast could be made to grow rapidly as judged by carbon dioxide production. This fact he thought to be due to a substance in the yeast extract which in addition to other known nutrients had to be supplied for the nutrition of the yeast cells. This new substance indispensable for the growth of yeast Wildiers called "bios." He found the substance to be soluble in water and 80 per cent alcohol but insoluble in ether. It was dialyzable and it was not precipitated by any of the ordinary precipitants including

* This work was carried out in connection with a fellowship for the study of Yeast Nutrition given by the Fleischmann Company.

¹ Wildiers, E., *La Cellule*, 1901, xviii, 313.

phosphotungstic acid. The substance was stable in acid solution but was destroyed by short boiling in dilute alkali. This instability was not uniform as he obtained very divergent results. Wildiers found "bios" to be absent from acid-hydrolyzed egg albumin, and from yeast ash.

Devloo² in 1906 claimed to have prepared "bios" in a state of purity from lecithin. Judging from his own statements, however, the material he prepared was in reality not very active in promoting yeast growth. His work will be referred to later in the discussion.

Pringsheim³ in 1906 showed that in the synthetic solution referred to by Wildiers there was unquestionably some growth of single yeast cells, though the growth was small. He sought to refute the work of Wildiers by saying that "bios" was nothing more nor less than protein material which theoretically should be obtained in most available form for yeast, from yeast itself. Evidently this argument stood, as in more recent years no mention of Wildiers' work has been found.

In this laboratory a study of the nutrition of yeast was undertaken with the intention of first studying solutions of known composition. In working with synthetic solutions it was also thought desirable to first obtain a pure culture of yeast grown on a synthetic medium if such a thing were possible. However, using the Lindner droplet method the single cell from which the pure culture was to be grown in such a medium could never be made to produce a colony large enough to be seen with the naked eye, and consequently such a culture was out of the question. Bearing in mind Wildiers' work, other experiments along the same line were performed with the result that Wildiers' observations were largely confirmed. The possibility presented itself that the substance which Wildiers called "bios" might be the same as the vitamine known to be contained in yeast. With this possibility in mind, a preparation of "activated" fullers' earth was obtained through the courtesy of Williams & Seidell and it was found to contain a substance which in small amounts would promote remarkably the growth of single yeast cells. The other work reported below is the logical outgrowth of this initial observation.

EXPERIMENTAL.

I. General procedure.—In preliminary experiments fairly uniform results were obtained by using merely a suspension of commercial pressed yeast for seeding, but for all the experiments

² Devloo, R., *La Cellule*, 1906, xxiii, 361.

³ Pringsheim, H. H., *Centr. Bakteriolog., 2te Abt.*, 1906, xvi, 111.

here reported a pure culture of baker's yeast was used. All experiments except under VI were conducted with a pure culture grown in this laboratory. In these experiments a pure culture was used which was kindly furnished by Dr. R. E. Lee of the Fleischmann Company. The results obtained on this culture in that study were also confirmed with the other culture.

In order to maintain a pure culture during the necessary manipulation most of the work was carried on inside a sterile cupboard made for the purpose. This glass cupboard was sprayed inside with dilute alcohol and closed generally a day or more previous to its use. The sliding glass door was opened during the work only enough to allow free movements of the hands of the experimenter.

The greatest difficulty in carrying out the experiments reported below, and in obtaining uniform results, lay in obtaining yeast in the right condition so that the colonies would break apart easily when a suspension was made and at the same time contain a large proportion of live cells. If, for instance, yeast which had grown one day was suspended, the yeast cells stayed in colonies and if a suspension was shaken hard enough to break the cells apart most of the cells were killed or their vitality was so lowered that they did not grow under the conditions of the experiment. In the case of yeast which has grown for a week or more at 30°C. or a shorter time at 37°C. the cells of colonies break apart into single cells easily, but due to autolysis, a considerable number of the cells are dead, and those which do grow, grow distinctly more in a solution containing ammonium salt as the only source of nitrogen than do cells when autolyzed material is not present. The autolyzed material of such a culture contained in a speck of yeast much smaller than a pin head when added to 25 cc. of solution can be detected by the increased growth of cells in the hanging drops. For the experiments reported the yeast used was grown 3 or 4 days at 30°C. and in most cases kept in the refrigerator a day or two before being used. In such a growth the cells often had some tendency to remain in clusters but it was much more easily worked with than younger yeast. The yeast at best seemed to vary somewhat in its properties when grown apparently under the same conditions. At times during the investigation the yeast was easier to work with than at other times. All the

yeast used in the experiments reported was grown on a rich malt wort obtained from a Fleischmann factory, and solidified with agar. When yeast was grown on a wort which had been autoclaved a long time or many times the growth of cells in a solution of mineral salts and sugar was still less than that reported in the case of the more vigorous yeast.

In the preliminary experiments various solutions were used, in which asparagine and ammonium lactate were used as sources of nitrogen, but in the experiments which are reported here the simpler salt $(\text{NH}_4)_2\text{SO}_4$ was used. The solution used consisted of the following substances dissolved in a liter of distilled water.

20	gm. cane sugar.
3	gm. $(\text{NH}_4)_2\text{SO}_4$
2	gm. KH_2PO_4
0.25	gm. CaCl_2
0.25	gm. MgSO_4

Yeast cells are not sensitive to small changes in hydrogen ion concentration; nevertheless, the phosphate buffer with the very small additions made served to control the acidity of the medium.

The solutions to be tested were always sterilized, ordinarily at 10 pounds pressure for 10 minutes before being seeded with yeast. In some cases the solutions were kept in the refrigerator 2 or 3 days after sterilization before the experiment was carried on. Bacterial contamination was carefully avoided in all material. When in preliminary experiments a "mineral salt solution" became contaminated yeast cells grew in the solution better than before contamination, although the solution was sterilized before being seeded with yeast. Hence it was necessary to avoid foreign organisms and the nature of the method made it possible to be sure that this factor was controlled.

When the yeast culture and the solution were in proper condition the seeding was carried out in the sterile cupboard mentioned above. A small amount of yeast was taken out of the culture and suspended in a test-tube containing about 10 cc. of sterile water. The amount of yeast put in approximated the size of a pin head, but could be estimated only after a little practice. The suspension was then slightly cloudy. A 1 cc. sterile pipette was introduced into the suspension and attached

to a rubber tube with a pinch-cock. The rubber tube contained a good cotton plug. By blowing through the tube and pipette a uniform suspension of the yeast was insured at the moment the pipette was filled. 1 cc. of the suspension was then put for trial into a flask containing the same amount of water as the solutions to be tested. After gently shaking the very dilute suspension a sterile steel pen was dipped into it and 25 drops were made on a cover-slip and immediately inverted on a hollow ground slide prepared with vaseline for sealing the chamber air-tight.⁴ The drops were then examined under the low power of the microscope to see if the seeding was about the right amount. If the seeding was much too great it was corrected by diluting the suspension with sterile water and if too small by using more of the suspension for seeding or adding more yeast to the suspension. Ordinarily there was no difficulty in obtaining the right amount of seeding as it is not necessary to have an exact and definite concentration of cells. The number of cells in the hanging drops can be varied by varying the time interval between shaking the solution and dipping the pen. If slightly too many cells are present in the drops when the pen is dipped immediately after shaking, waiting a moment before dipping the pen will give the desired results. When the correct seeding was found, each solution to be tested was seeded immediately in the same way. In this way the yeast to be seeded remained in the distilled water only a few minutes.

At least 50 hanging drops on two slides were made in the way described from each solution to be tested. By using a standard pen the drops were of fairly uniform size and by having the solution as cool as possible evaporation was avoided. The contents of each drop which contained one single cell, two cells joined together, or one single and one double cell or two of each and no more, were recorded on squared paper. In order to increase the number of observations a single cell with a small bud was counted as a single cell and a double cell with a bud was counted as a double cell. This did not change the trend of the results as all solutions were treated alike in this manner and no conclusions are drawn from differences unless they are large.

⁴ Thanks are due Dr. Lee for his suggestion of the hanging droplet method of study.

The individual variations of the yeast cells are quite large as will be seen in the results below. Many observations showed that yeast cells grow practically as well whether there are one or two cells in a drop provided the growth is not carried too far. After the contents of each slide had been recorded the slide was put into an incubator at 30°C. and the time of incubation recorded. In this manner eight satisfactory slides of 25 drops each representing four solutions could be made, and the contents recorded in an hour.

After the cells had been growing 5 or 6 hours all the slides were examined. Slight growth had taken place in this time and the records of the contents of the drops were verified and the growth was recorded. In case any cells were overlooked they were noted and if more than two colonies were located in one drop, the particular drop was excluded from consideration. At the end of 20 to 24 hours the drops were examined and counts again made. If a colony could not be counted because of the presence of some debris (for instance flakes from the steel pen) it was excluded from consideration. This was also true if it could not be determined with a good degree of certainty whether a certain colony was produced by a single or double cell or both, both of which might have been in the same drop. Ordinarily, however, few drops were excluded and practically all the original cells were considered. No colonies were excluded because of irregularities of growth. In case the colonies were small the count could be accurate; larger colonies were less accurately determined as the colonies grow to some extent in three dimensions. Estimations of the larger colonies were made by counting a portion of the colony and estimating the number of times it was contained in the whole. An effort was made to make the estimates on large colonies conservative.

Often the contents of the drops were watched for several days but the results are not reported unless they have some added significance. In some experiments there were practically no single cells present, and in others few double cells were present, consequently only one kind is reported in the experiments, provided there were sufficient numbers to warrant drawing conclusions. The growth of single and double cells was always parallel.

II. Protein-Free Milk.—Osborne and Mendel have used protein-free milk extensively as a source of the water-soluble vitamine.

This experiment was performed to find out if the substance promoting the growth of yeast is also present in such material. 200 cc. of milk were made "protein-free" following the method of Osborne and Mendel.⁵ The filtrate which was water-clear was evaporated to dryness on a water bath in a current of air. It was further dried by alcohol and ether treatment and kept in a vacuum desiccator. This material was extracted three times with warm ether and three times with a like quantity of warm 95 per cent alcohol. The ether extract which was very slight in amount was digested with water and made up to the original volume of

TABLE I.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 1 cc. H ₂ O.....	20	12	15	4	8
II. 25 cc. control solution + 1 cc. ether extract of protein-free milk.....	14	4	8	4	6
III. 25 cc. control solution + 1 cc. alcohol extract of protein-free milk.....	18	10	180	36	108

the milk. The alcoholic extract which was much larger in amount was also digested with water and made up to the original volume of the milk.

The solutions to be tested were made up as indicated in Table I, sterilized, and seeding carried out in the manner described. Growth at the end of 22 hours was recorded. The results show plainly that the growth stimulant is present in the alcohol fraction of protein-free milk.

III. Wheat Germ.—Wheat germ has been used as a source of the water-soluble vitamine by McCollum and his coworkers as well as others.

⁵ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, pts. i, ii, 1911.

In an experiment not performed specifically for this purpose and only part of which is reported (Table II), it is shown that wheat germ may be used as a source of the substance promoting the growth of yeast.

Wheat germ was extracted three times with a good quantity of hot 95 per cent alcohol, the extract was filtered, and the filtrate evaporated to dryness. The residue was digested as completely as possible in such a quantity of water that 1 cc. of the solution was equivalent to 75 mg. of the original wheat germ. After filtration 2 cc. of the solution were used for the experiment.

The results recorded in Table II were obtained at the end of 22 hours growth. There is evidence of a considerable amount of the yeast growth substance present in wheat germ.

TABLE II.

Composition of solution.		Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.	25 cc. control solution + 2 cc. H ₂ O.....	20	2	3	2	2.5
II.	25 cc. control solution + 2 cc. alcoholic extract of wheat germ.	18	8	135	54	91

IV. *Lactose*.—McCollum and Davis⁶ found in feeding experiments, in which they used 20 per cent of Kahlbaum and Merck's lactose, that the lactose contained the water-soluble vitamine as an impurity. We ran tests to ascertain if the yeast growth substance was likewise present in lactose as an impurity.

10 gm. of Kahlbaum's lactose were extracted with 95 per cent alcohol continuously for 6 hours. The alcohol was cooled, filtered free from lactose crystals, and evaporated to dryness on the water bath. The residue was then dissolved in 10 cc. of water. 5 cc. of this solution were used for each of the experiments reported below. The two experiments are used together as neither one by itself was absolutely conclusive, due to the low vitality of the yeast used. The two experiments were conducted in the same way except that the yeast used in the second case had been in the refrigerator one day longer and 2 cc. of a suspension instead of 1 cc. were used for seeding.

⁶ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 183.

The results of growth at the end of 22 hours of both double and single cells are reported in Table III and show definitely the presence of a small amount of the growth-promoting substance in Kahlbaum's lactose.

V. Pancreas Tissue.—Eddy⁷ has found the water-soluble vitamin to be present in fairly large quantities in pancreas tissue.

This experiment was performed to determine whether the yeast growth substance is also present in pancreas tissue, pancreatin U. S. P. being used as a convenient source. 2 gm. of pancreatin were extracted directly with warm ether and filtered through a good filter. The residue was then extracted in like

TABLE III.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 5 cc. H ₂ O..	22	3	12	7	9
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose.....	29	12	50	18	36
-----	Single cells considered.	-----	-----	-----	-----
I. 25 cc. control solution + 5 cc. H ₂ O.	8	4	10	3	6
II. 25 cc. control solution + 5 cc. alcoholic extract of lactose.....	9	3	36	10	23

manner with warm 95 per cent alcohol and filtered. Each filtrate was evaporated to dryness and dissolved in 20 cc. of water. 1 cc. was used in the experiments which was equivalent to 0.1 gm. of pancreatin.

Table IV gives the results of a 22 hours growth following the usual procedure and shows the presence of a considerable amount of the yeast growth substance.

VI. Hydrolyzed Casein.—The purpose of this experiment was to test the possibility that the growth-promoting substance of yeast might be some of the amino-acids derived from an "adequate" protein like casein.

⁷ Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.

5 gm. of casein Merck (Hammarsten) were digested with 25 per cent H_2SO_4 for 14 hours using a reflex condenser. The bulk of the acid was removed with $Ba(OH)_2$ and the solution brought to very slight acidity. This was made up to 500 cc. volume and sterilized on two occasions at 10 pounds pressure for 10 minutes. When ready for use, to a portion was added crystalline tryptophane up to 1.5 per cent of the original casein digested. The tryptophane had been prepared from casein by the Hopkins method. It is obvious from the previous experiment why pancreatin was not used to digest the casein. For purposes of comparison solutions were used which were known to contain the growth-promoting substance, an alcoholic extract of an alkali extract of "activated" fullers' earth being used as a source of the

TABLE IV.

Composition of solution.	Double cells consid- ered.	Num- ber grow- ing.	Maxi- mum growth.	Mini- mum growth.	Average growth.
I. 25 cc. control solution + 1 cc. H ₂ O.....	14	8	10	4	5.6
II. 25 cc. control solution + 1 cc. ether extract of pancreatin.....	13	6	15	5	9.2
III. 25 cc. control solution + 1 cc. alco- holic extract of pancreatin.....	15	13	280	80	187

growth-promoting substance. Each cc. added contained 1 mg. of actual material. Because a considerable amount of the casein digest was added in this experiment additional phosphate buffer was used to control the acidity.

Table V gives the results for the first 5 hours growth and Table VI for the first 22 hours.

Noting the fact that the growth in Solution IV had slowed up it was thought desirable to watch it another day.

At the end of 48 hours the growth of the three colonies in each solution which had shown maximum growth in 22 hours were counted or estimated (Table VII).

In Solution IV the initial growth was most rapid as would be expected, but later was markedly retarded. The results clearly indicate that as the yeast grows in the solution containing both

TABLE V.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 11 cc. H_2O	17	2	3	3	3
II. 25 cc. control solution + 1 cc. 10 per cent KH_2PO_4 solution + 1 cc. fullers' earth extract.....	16	11	6	3	4.0
III. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. H_2O	13	9	5	3	3.6
IV. 25 cc. control solution + 10 cc. casein digest + tryptophane + 1 cc. fullers' earth extract.....	15	12	10	3	6.1

TABLE VI.

Solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I.....	17	6	9	4	5.8
II.....	16	15	95	12	58.0
III.....	13	12	10	3	8.9
IV.....	15	14	46	17	32.0

TABLE VII.

Solution.	Average of the three maxima.	
	22 hours.	48 hours.
I.....	8	11
II.....	75	283
III.....	10	13
IV.....	43	45

casein digest and the growth-promoting substance, something harmful is formed which is not initially present. This may throw some light on the observation of Effront as well as others that too complete hydrolysis of protein injures it as a yeast food.

Aside from this question the experiment shows that the growth-promoting substance is not one of the commoner amino-acids known to be contained in an acid digest of casein.

VII. Adsorption from Malt Wort by Fullers' Earth.—Seidell^s has found that the water-soluble vitamine is adsorbed nearly quantitatively from autolyzed yeast filtrate by certain varieties of fullers' earth and is thus removed from the bulk of the original material.

As has been mentioned it has been found that such a preparation contains the substance which stimulates yeast growth in relatively large quantity. The experiment reported below was performed to confirm this fact and to determine if there is anything unadsorbed by the fullers' earth which may take the place of the adsorbed material.

Fresh malt wort obtained from one of the Fleischmann factories was evaporated on the steam bath to about one-half its original volume. It was filtered and a portion diluted 1:20, while a 25 cc. portion was shaken continuously with 1 gm. of fullers' earth (Eimer and Amend) for 1 hour. The fullers' earth was filtered off, the filtrate diluted 1:20, and the fullers' earth washed once with water. The fullers' earth was then extracted by shaking 10 minutes with saturated $\text{Ba}(\text{OH})_2$, filtered, and the barium removed with H_2SO_4 . The resulting solution was diluted so that 2 cc. were equivalent to 3 cc. of the diluted wort. This experiment was carried on with the pure culture furnished by the Fleischmann Company using the general method previously described.

Table VIII gives the results of 6 hours of growth.

At the end of 24 hours, due to the large number and size of the colonies, only the three in each solution which had shown maximum growth in 6 hours were considered (Table IX).

One effect is observed here but not recorded in any other experiments. The yeast used for seeding had remained in the

^s Seidell, A., *Bull. Hyg. Lab., U. S. P. H.*, 1916, xxxi, 364.

refrigerator a week, during which time autolysis had evidently taken place. A large percentage of cells was not able to grow even in the good medium. Furthermore, the growth of those which did grow in the control solution was greater than in other

TABLE VIII.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 5 cc. H ₂ O.....	25	10	4	2	2.4
II. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. H ₂ O.....	22	10	8	2	3.2
III. 25 cc. control solution + 3 cc. diluted fullers' earth-treated wort + 2 cc. alkaline extract of fullers' earth.....	19	11	10	2	5.5
IV. 25 cc. control solution + 3 cc. original untreated diluted wort + 2 cc. H ₂ O.....	23	15	13	2	5.6

TABLE IX.

Solution.	Number considered.	Number growing.	Growth of the three maxima.			Average growth of the three maxima.
I.....	25	13	2	16	10	15
II.....	22	11	51	40	42	44
III.....	19	12	180	300	180	220
IV.....	23	12	200	375	300	292

experiments, due evidently to the autolyzed material added with the live cells. That the effect is not due to the vigor of the yeast was shown by the fact that the same yeast was used in a previous unreported experiment, where, before the slight autolysis had taken place, the cells had grown much less than in this experiment.

The foregoing experiment shows that the growth-promoting substance is taken out by shaking a solution with fullers' earth, just as vitamine is known to be. Probably it can be removed even more quantitatively by observing the right conditions of acidity, quantity of earth used and time of shaking; conditions which as yet have not been definitely determined.

VIII. Phosphotungstic Acid Precipitation.—Since the earliest work of Funk, precipitation by phosphotungstic acid has been used as a means of purification of the vitamine which prevents beri-beri. Wildiers found the so called "bios" not to be precipitated by phosphotungstic acid. The conditions of precipitation which he used were not definitely stated. Consequently, the experiment was repeated to determine if under proper conditions the yeast growth substance can be precipitated with phosphotungstic acid.

20 gm. of "activated" fullers' earth were shaken with 200 cc. of saturated $\text{Ba}(\text{OH})_2$ for 5 minutes and filtered. The filtrate was brought with H_2SO_4 to very slight acidity, filtered, and the filtrate concentrated *in vacuo* to about 40 cc. and filtered from organic residues. The filtrate was divided into two equal portions. To one portion was added 50 per cent H_2SO_4 until it contained 5 per cent acid, and to it was added as a precipitant altogether 10 cc. of a 20 per cent solution of phosphotungstic acid dissolved in 5 per cent H_2SO_4 . This was a slight excess of phosphotungstic acid. It was allowed to stand over night. The precipitate of phosphotungstates was filtered off and washed thoroughly with 5 per cent H_2SO_4 containing 0.5 per cent of phosphotungstic acid. The filtrate was made slightly alkaline with $\text{Ba}(\text{OH})_2$ and filtered, the precipitate being washed thoroughly with half saturated $\text{Ba}(\text{OH})_2$. The filtrate was then made very slightly acid, filtered, and after dilution to 200 cc. was used in the experiment as the "phosphotungstic acid filtrate." The phosphotungstates were decomposed with five 20 cc. portions of saturated $\text{Ba}(\text{OH})_2$ grinding each time in a mortar. The material was in contact with the alkali a total of over an hour and the last two portions of alkali were warmed gently with the precipitate. The material was filtered and the precipitate washed on the filter with saturated $\text{Ba}(\text{OH})_2$. The filtrate was made very slightly acid with H_2SO_4 and again filtered.

This was also diluted to 200 cc. and used in the experiment as "decomposed phosphotungstates." The original untreated solution was likewise diluted and equivalent amounts (2 cc.) of each solution were used. Each portion added was equivalent to 0.1 gm. of the "activated" fullers' earth. Each of the solutions had been heated in the autoclave a total of 10 minutes at 10 pounds pressure.

Hanging drops were made in the usual way. Table X shows the results at the end of 24 hours.

To get the best quantitative interpretation of the results the number growing in the solution should be considered as well as

TABLE X.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 2 cc. H ₂ O.....	16	3	5	2	3.3
II. 25 cc. control solution + 2 cc. original untreated extract.....	15	13	70	14	35.
III. 25 cc. control solution + 2 cc. "phosphotungstic acid filtrate"	18	4	33	2	25.
IV. 25 cc. control solution + 2 cc. "decomposed phosphotungstates".....	14	10	70	11	51.

the average growth. Solution IV was most active, Solution II next in activity, and III much weaker as shown by the small percentage which grew. The "decomposed phosphotungstates" show greater activity than the original extract. Later the explanation for this result was found in the effect of acid and alkali on the material and will be discussed later. The activity of all the solutions except Solution I was considerably less than would be expected from the amount of earth used, judging by other experiments. Possibly some activity was lost when the original material was filtered after concentration *in vacuo*. That the substance is precipitated by phosphotungstic acid cannot be doubted. A similar result to that reported was obtained in other

experiments, the precipitation being more incomplete with more dilute solutions.

IX. Heat Stability.—Considerable divergence of opinion exists as to the degree of heat stability of the water-soluble vitamine. The more recent work indicates that in foods and in ordinary preparations it is quite stable to heat, not being destroyed at boiling temperature and withstanding considerable heating under pressure. No doubt preparations vary depending on various factors.

An alcoholic extract of dried yeast was tested for the heat stability of the substance promoting the growth of yeast. To each of two flasks were added 6 cc. of a solution of yeast extract, which had been prepared by extracting dry yeast with 95 per cent

TABLE XI.

Composition of solution.	Double cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 6 cc. H ₂ O.....	12	3	4	3	3.3
II. 25 cc. control solution + 6 cc. autoclaved extract.....	20	12	6	3	4.3
III. 25 cc. control solution + 6 cc. untreated extract.....	11	7	10	3	5.6

alcohol, and to a third flask 6 cc. of distilled water. Each of the samples of yeast extract was equivalent to 0.3 gm. of dry yeast. One of the portions of extract was heated in the autoclave for 30 minutes at 15 pounds pressure and brought to its original weight with distilled water. To each of the three flasks were then added 25 cc. of the control solution and they were sterilized as usual for 10 minutes at 10 pounds pressure. After 6 hours growth in hanging drops results were obtained as recorded in Table XI.

At the end of 23 hours estimations were made on the three colonies in each solution which in 6 hours had shown the maximum growth (Table XII).

The substance promoting the growth of yeast in the condition in which it was used was partially destroyed by heating under 15 pounds pressure for 30 minutes.

X. Treatment with Acid or Alkali.—The water-soluble vitamin has been found to be quite stable in acid solution even at the boiling temperature. Vedder and Williams⁹ found it to be markedly changed in its physiological properties by acid treatment and suggested a hydrolysis as a possible explanation. On the other hand it has been found to be unstable in alkaline solution. On this point there has been a wide divergence of results. At least one worker has reported its destruction by as weak a base as ammonia. McCollum and Simmonds¹⁰ found that as prepared from wheat germ it was destroyed by short boiling with very dilute alkali. Williams and Seidell¹¹ have found the preparation extracted from "activated" fullers' earth to be quite stable to alkali, though the alkali alters it in some way without destroying its property of preventing polyneuritis in pigeons. Again a hydrolysis was suggested to explain the change. Recent

TABLE XII.

Solution.	Growth of three maxima.			Average of three maxima.
	9	8	10	
I.....	9	8	10	9
II.....	160	180	150	163
III.....	400	320	200	307

work by Daniels and McClurg¹² has shown that as the vitamin exists in foods it is not readily destroyed by cooking under pressure with very dilute alkali.

The study of the behavior of the growth-promoting substance of yeast toward acid and alkali has proven to be a complicated problem, due apparently to the interference of toxic substances formed. This study is therefore not complete although a large number of experiments has been performed. An indication of the trend of the results will be given.

The fullers' earth preparation has been found to be quite stable in alkaline solution, confirming the observations of Williams and

⁹ Vedder, E. B., and Williams, R. R., *Philippine J. Sc.*, 1913, viii, 180.

¹⁰ McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 88.

¹¹ Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 432.

¹² Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, xxxvii, 201.

Seidell¹¹ for the vitamine. There seems no doubt from our experiments that this material when acted on by acid or alkali is changed (presumably by hydrolysis) to a form which is more immediately available for the yeast. The treatment does not change the total amount of yeast which is produced in 2 or 3 days but makes the initial growth for 24 hours as much as three times as fast. These results are in accordance with the observations mentioned above on the supposed hydrolysis. This, it is thought, adequately explains the result obtained in the experiment on phosphotungstic acid precipitation as both the "filtrate"

TABLE XIII.

Composition of solution.	Single cells considered.	Number growing.	Maximum growth.	Minimum growth.	Average growth.
I. 25 cc. control solution + 25 cc. H ₂ O.....	25	3	6	4	4.7
II. 25 cc. control solution + 1 cc. H ₂ O + 1 cc. ether extract of egg yolk.....	17	5	14	7	9
III. 25 cc. control solution + 1 cc. H ₂ O + 1 cc. alcoholic extract of egg yolk.....	29	15	120	29	68
IV. 25 cc. control solution + 1 cc. ether extract of egg yolk + 1 cc. alcoholic extract of egg yolk....	26	20	200	23	59

and "decomposed phosphotungstates" had stood in 5 per cent H₂SO₄ for some time.

The results obtained on various other preparations would suggest that the substance promoting the growth of yeast, as it exists in yeast and wheat germ (alcoholic extracts), is not very unstable in the presence of alkali. However, a previous exhaustive extraction with dry ether seems to render the alcoholic extract obtained afterward more labile to alkali. We have some evidence that this is true also of the fullers' earth preparation. It seems to be unstable to alkali when the material is extracted with ether before alcoholic extraction. This will be referred to later.

XI. Fat-Soluble Vitamine.—It was thought desirable to find out whether the fat-soluble vitamine has any effect on yeast growth.

The yolk of hard boiled egg was selected as a source as it contains both water-soluble and fat-soluble vitamins in good quantity. The yolk was removed from a hard boiled egg, partially dried in a desiccator, then ground with plaster of Paris, and thoroughly dried over calcium chloride in a vacuum desiccator. The material was continuously extracted for 10 hours with sodium-dried ether, taking extra precaution to avoid the presence of moisture. It was then extracted for 3 hours with 95 per cent alcohol. Each extract was evaporated to dryness, digested as completely as possible in water, and filtered. The solutions were made so that 1 cc. was equivalent to 0.1 gm. of dry egg yolk.

The results obtained after 22 hours growth are given in Table XIII. The effect of the fat-soluble vitamine, if there is any effect, is not marked either as a substitute for the alcohol-soluble substance, or as complementary to it. If the yeast has a requirement for the fat-soluble vitamine it is exceedingly minute.

DISCUSSION.

In the preceding experiments it has been shown that the substance promoting growth of yeast occurs in the same materials as those in which vitamine has been found; namely, protein-free milk, wheat germ, lactose, yeast, egg yolk, and pancreatin. The substance is none of the commoner amino-acids contained in an acid digest of casein with tryptophane added. It has the same properties of solubility, precipitation by phosphotungstic acid, adsorption in fullers' earth, heat stability, and behavior toward acid and alkali, as nearly as we know those properties, as the water-soluble vitamine, and in addition the two substances so far as known have no divergent properties. A warning should be given that the results reported here in different experiments are not comparable one with another and were *not* designed to show quantitative comparisons of different materials. As far as we are able to judge, however, taking into consideration the apparent hydrolysis factor which probably is important, as well

as the method of extraction, those substances which are richest in vitamine are richest in the yeast growth substance. The fullers' earth preparation is richest of any of the sources tested. Wheat germ and yeast are poorer than the fullers' earth preparation and are of the same order as has been found to be true of the vitamine content in animal experiments. Milk is still poorer in the yeast growth substance as is also true of its vitamine content.

From the cumulative evidence offered we believe we are justified in concluding that as far as present knowledge is concerned the substance or substances which stimulate the growth of yeast is or are identical with the substance or substances which in animal nutrition prevent beri-beri or polyneuritis.

If this conclusion is true the water-soluble vitamine must be a most fundamental nutritional requirement playing an indispensable rôle for a great variety of organisms. It has not before been shown conclusively to be necessary for the nutrition of any members of the plant kingdom. Some suggestions have been made as to its possible importance in the nutrition of bacteria. We have accidentally observed that some species of molds are apparently able to produce it. Pacini and Russell¹³ have shown that typhoid bacilli must be able to produce it.

As it is apparently possible to cause a single yeast cell to produce from 20 to several thousand cells in 24 hours by varying the vitamine content of the culture medium, we hope the method may be valuable both as a qualitative and ultimately as a quantitative test for vitamine. Work is now being done to make the test more applicable quantitatively and will be reported upon later. As a qualitative test extremely small amounts may be detected. The most serious obstacle in the purification and study of vitamines has been the difficulty of obtaining sufficient material to work with and test. Yeast can be used to test for the water-soluble vitamine and only infinitesimally small amounts need be used for testing. The accompanying photomicrographs (Figs. 1 and 2) show the effect of the addition of 0.5 mg. of actual crude material to about 30 cc. of control solution and subsequent incubation for 24 hours. The drops in which the cells were growing weighed approximately 0.03 mg. each and contained therefore

¹³ Pacini, A. J. P., and Russell, D. W., *J. Biol. Chem.*, 1918, xxxiv, 43.

about one millionth of the 0.5 mg. of total material added. The material added was crude, being obtained simply by adsorption on fullers' earth from a malt wort and subsequent extraction with alkali. The test can be easily applied after a little practice. The vitamine if present in sufficient but very small quantity can be detected in the course of 4 or 5 hours.

Already the method has contributed something in the way of additional information in connection with the apparent hydrolysis for which the evidence is now more certain.

It seems that we have at least a partial explanation for the divergence of results in stability of the vitamine toward alkali, in the effect of previous ether extraction. In recent work McCollum and Simmonds¹⁰ found the vitamine to be very unstable toward alkali. The material they used was previously extracted for 18 hours with ether. Voegtlin and Lake¹⁴ observed that meat which had been freed from fat lost its anti-neuritic properties under the influence of heat and alkali much more easily than meat with the fat present.

The difficulty of obtaining pure materials and the likelihood of wrong inferences drawn from work with impure material is emphasized by our work. In earlier work on yeast, yeast no doubt grew better in "synthetic" media than it would have if the asparagine, ammonium tartrate, sugar, etc. used had been purer. Devloo's results on the preparation of "bios" from lecithin parallel the findings of an early investigator who cured polyneuritis on "pure" lecithin.

At this point I wish to thank Professor F. C. Koch for helpful suggestions and interest throughout the work and for his generosity in providing equipment.

CONCLUSIONS.

1. A substance of unknown nature, which is a constituent of yeast, is necessary in addition to the ordinary nutrients for the nutrition of yeast cells.
2. This substance (or substances) based upon identical occurrence and various properties is concluded to be identical with the beri-beri-preventing vitamine.

¹⁴ Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918-19, xlvii, 558.

3. The fat-soluble vitamine apparently has no effect on yeast growth.

4. The growth of single cells of yeast may be used as a simple biological test for vitamine, and it is hoped may be used to advantage in quantitative studies.

EXPLANATION OF PLATE.

FIG. 1. Growth produced by a single cell in 24 hours in "synthetic" solution.

FIG. 2. Growth produced in 24 hours under identical conditions except for the addition of one part in 60,000 of crude vitamine-containing material (cf. p. 484).



FIG. 1.



FIG. 2.

(Williams: Vitamine requirement of yeast.)

RELATIONSHIP OF THE PANCREATIC ENZYMES.

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Our idea in presenting this paper is not so much that the basic facts are new as that there is a real need for exact data on mixtures such as we have described. No comparative study of the various enzymes of the pancreas in relation to each other has been made with samples of known origin and composition. The methods used in preparing these samples are simple, practical, and capable of rendering representative mixtures which approach conditions existing in the body.

Attempts were also made to increase the activity of these enzymes to a maximum by means of sodium chloride, duodenal tissue, and fresh bile. Sodium chloride was employed because we have found it the most satisfactory means of preventing loss of diastasic power in the pancreas while drying. The duodenal mucosa and the bile are close associates of the pancreatic secretion and are generally considered activators of the proteolytic and lipolytic enzymes, respectively.

Starting then from these well known points, the relationship of the enzymes with and without activation becomes of interest. For control purposes, whole glands as well as duodenal tissue and fresh bile were prepared in addition to the mixtures, and tested along with the other samples.

Hog glands were selected because the omnivorous hog shows closer digestive relationship to the human race than do herbivorous animals such as cattle and sheep, and also because the large majority of commercial pancreatic preparations are derived from the hog. The glands were taken at random from the Chicago abattoir and represent hogs raised in the middle west. The pancreases, duodenums, and bile were collected from separate animals.

The duodenal tissue from hogs is always acid in reaction, while the bile is neutral or slightly alkaline. All the pancreatic glands which have come under observation in this laboratory during the past 8 years, amounting to several hundred lots and representing millions of hogs, were acid to litmus. The same is true of the cattle and sheep pancreases examined. It is certain, therefore, that the normal reaction of the pancreas is acid. Attention has been called to this fact in earlier communications¹ and the present statements may be regarded as additional and conclusive evidence.

The entire pancreatic glands were carefully removed immediately after slaughter of the animals, trimmed free from connective and other adherent tissue, and finely minced. A dozen or more of the pancreases from each lot were transferred, while still retaining the animal heat, to the vacuum oven and desiccated whole without disturbing the fatty tissue and protecting membranes surrounding the gland itself. This was done to eliminate the disturbing action of mincing and to minimize the possibility of atmospheric influence. These glands, therefore, represent very closely the actual condition existing in the pancreas of the living animal.

The duodenums, consisting of the first 12 inches of the small intestines immediately below the pyloric valve, were likewise removed and freed from adipose and connective tissue, care being taken to discard all traces of pancreatic structure. The tubes were cleansed by means of running water, before mincing. It may be mentioned here that in hogs the opening of the bile duct is about 2 inches and that of the pancreatic duct 4 to 5 inches below the pylorus. The bile was obtained directly from the gall bladders. In each set of experiments the mixtures were made from the same batch of minced material by weighing out the ingredients and mixing thoroughly, first by hand and then by running the mixture through a small meat chopper. The various preparations were spread on agate trays each holding 200 gm. or more and dried *in vacuo* at temperatures ranging from 30–40°C. After desiccation, the material was extracted with petroleum ether in Soxhlet extractors and powdered to 60 mesh.

¹ Long, J. H., and Fenger, F., *J. Am. Chem. Soc.*, 1915, xxxvii, 2213; *ibid.*, 1916, xxxviii, 1115.

In the tabulations will be found the date of collection and the number of glands and other material employed; also the loss in moisture, petroleum ether-soluble material, and the yield of desiccated fat-free substance. All enzymic determinations were made on the powdered samples.

Methods.

The methods employed in measuring the activity of the samples were mainly of a routine character. Strict care was taken to make them uniform so that in every case measurement of activity was comparable.

The diastasic activity was a measure of the gm. of anhydrous potato starch which 1 gm. of enzymic powder will change to dextrin, colorless to iodine, in 10 minutes at 40°C. The method was that outlined by Johnson,² and the reported values are in all cases the mean of at least three independent and closely agreeing checks on the sample.

The fat-splitting activity was measured by the cc. of 0.10 N NaOH required to neutralize the acid formed from the action of 0.100 gm. of the enzymic powder on 10 cc. of emulsified olive oil. The emulsion was formed by neutralizing the oil to phenolphthalein with 0.25 N NaOH. When a large enough quantity of oil for each series is taken and the mixture with NaOH is thoroughly shaken, an emulsion is formed which holds very well and by neutralizing the whole quantity for a series, a uniform sample is obtained.

The mixture of powder and oil was digested for 2 hours at 40°C. in a water bath, shaking every 15 minutes. At the end of digestion, 25 cc. of neutral alcohol and 5 cc. of acid-free ether were added and the mixture titrated with 0.10 N NaOH using phenolphthalein as indicator. For control the same procedure was followed except that the enzyme was killed by boiling the powder in a minimum amount of water for 15 minutes. In every case correction for this blank has been made. This method has been described by Wohlgemuth.³ The action of lipase on olive oil seems to be representative of this group of ester-splitting ferments.

² Johnson, W. A., *J. Am. Chem. Soc.*, 1908, xxx, 798.

³ Wohlgemuth, J., *Grundriss der Fermentmethoden*, Berlin, 1913, 102.

Two methods of measuring tryptic activity were used. One, the well known Fuld-Gross method, needs no outlining in these pages, while the other is almost as familiar. This second is that of measuring the amino-acids formed by digestion with the enzyme. It having been found by one of us⁴ that the optimal activity of trypsin is to be obtained in digestion of that enzyme with blood fibrin in a mixture of di- and monosodium phosphate, this mixture was used as follows: 900 cc. of 0.067 M disodium phosphate and 100 cc. of 0.067 M monosodium phosphate (prepared according to Sørensen⁵) were mixed and 25 cc. of this were taken for each digestion, mixed with a weight of dried 40 mesh blood fibrin, equivalent to 1 gm. of protein, and 25 cc. of water. This mixture was digested for 3 hours at 40°C., shaking every 15 minutes.

After digestion each mixture was treated with 1 gm. of barium chloride and barium hydroxide to strong alkalinity (approximately 20 cc. of saturated Ba (OH)₂), made to 100 cc. and after the precipitated proteins and phosphate had settled, a 50 cc. aliquot was filtered off, made neutral to litmus, 10 cc. of neutral formaldehyde were added, and the mixture was titrated with 0.25 N NaOH using phenolphthalein as indicator. This titration represents, when corrected for its control, the equivalent of mono-amino-acid nitrogen formed in one-half of the digestion.

The controls here, as in other cases, consist in parallel determinations in which the enzyme was "killed" by boiling 15 minutes. These blanks were very small, in no case amounting to more than 1.2 cc. of 0.25 N NaOH, an average value being 1.0 cc., and represent both the action of the buffer solution on the proteins and the acidity of and amount of amino-acid present in the enzymic powder itself. In each case the values quoted in Table I have been corrected for this blank and represent real enzymic activity.

The fibrin used in these experiments was prepared in this laboratory from fresh beef blood. The casein for the Fuld-Gross tests was prepared by one of us from fresh, commercial fat-free milk by a series of precipitations until free from salts. The purified precipitate was washed rapidly with alcohol and ether and powdered to 40 mesh.

⁴ Long, J. H., and Hull, M., *J. Am. Chem. Soc.*, 1917, **xxxix**, 1051.

⁵ Sørensen, S. P. L., *Biochem. Z.*, 1909, **xxi**, 131.

All the samples prepared for this series of experiments were tested according to the well known colorimetric method of measuring pH values. 0.10 gm. of the powders was mixed intimately with 30 cc. of CO₂-free water and filtered after standing 15 minutes. 10 cc. of this filtrate were used for the determination, with neutral red and *p*-nitrophenol as indicators. Mixtures of 0.067 M mono- and disodium phosphate were used as standards. The straight pancreas samples showed pH values ranging between 6.11 and 6.14 while that of the duodenal tissue was found to be 6.21. We were unable to make an accurate determination of the bile, due to its strong color which masks the reaction. It has been shown by one of us,¹ however, that the pH value for bile when measured by the potential cell, lies between 7.04 and 7.21. The reaction consequently is neutral or very faintly alkaline. All the mixtures in the tabulation, including those containing bile, showed pH values between the limits of 6.11 and 6.21, and are, therefore, acid in reaction.

Tests were also made on the reaction of the buffer solution used in the fibrin digestion experiments with trypsin. The two phosphate solutions after mixing and dilution with water to 50 cc. showed a pH value of 8.02. When this same dilution was mixed with enzymic powder and fibrin exactly as for digestion and due time allowed for the establishment of equilibrium, the pH value of the filtrate was found to be 7.40. The pH value of the liquids after digestion for 3 hours at 40°C. ranged from 7.30 for straight pancreas powder, to 7.18 in cases where the pancreas and duodenal mixtures were employed. All the other samples come within these two limits. The alkalinity of the solution naturally decreases inversely to the activity of the proteolytic enzyme and the amounts of amino-acids formed during digestion.

Tabulation.

It will be seen from the tabulated results that the whole normal pancreas possesses high diastasic, considerable lipolytic, and some proteolytic power. The diastase is present in fully activated form ready for immediate use, while the lipase and trypsin need the stimulating effect of the bile and duodenal mucosa respectively, before reaching maximum activity.

Both mincing and drying lower the diastasic power of the fresh gland materially, while the proteolytic and lipolytic enzymes seem more resistant to mechanical interference. The quicker the moisture is removed from the minced material the smaller will be the loss in diastase. Thus, vacuum drying at blood heat is less destructive than desiccation under atmospheric pressure, and especially at higher temperatures. Drying in air, for instance, at 50°C. reduces the diastase power still further, indicating the sensitivity of the enzyme to heat.

The mincing process breaks up many of the pancreatic cells, causing the liquid or semiliquid contents to be mixed. This radical treatment completely upsets the glandular equilibrium and it is only logical to expect enzymic disturbances to occur. The diastase, being the most sensitive of the enzymes, naturally suffers the most. If the gland is left intact and dried *in vacuo* at low temperature, the equilibrium is not disturbed and no enzymic deterioration is evidenced.

The loss in diastasic activity may be prevented by the addition of a small amount of sodium chloride, preferably in combination with bile, to the freshly minced material before desiccation. This also applies to pancreatic mixtures containing duodenal mucosa and activated trypsin. The influence of the salt is of a protective or stabilizing nature rather than a true activation process, since the diastasic power is not increased materially beyond that of the undisturbed whole gland. Sodium chloride seems to have a slightly stimulating effect on the lipolytic enzyme also, while it does not influence the trypsin one way or the other.

In the living animal, the stabilizing action of sodium chloride is apparently not needed until the pancreatic juice enters the duodenum. It has been shown in an earlier paper that the juice obtained from the entire fresh hog pancreas by centrifugal force contains only 0.012 per cent chlorine, equivalent to 0.02 per cent sodium chloride. When allowance is made for the salt present in the natural blood supply of the pancreas, it will be seen that the true pancreatic secretion is practically free from chlorides. The sodium chloride is encountered in the duodenum where it is present as a natural constituent of the bile. The samples of bladder bile employed in these experiments contained on an average 0.33 per cent of sodium chloride. Since, in this case, the bile duct enters

the duodenum above the pancreatic outlet, the presence of sodium chloride is always insured during active digestion. The hydrochloric acid present in the gastric content gradually combines with the organic sodium salts in the bile, furnishing additional sodium chloride which likewise tends to maintain the diastasic power. The results shown in Experiment 4 indicate that the sodium chloride as present in the bile is not sufficiently available to completely offset loss in the diastasic power during the desiccation processes. The mucin of the bile evidently retards the action of sodium chloride to a considerable extent. If a little salt is added to the pancreas and bile mixture, as in Experiment 5, there is a small increase in diastasic action even over that present in the whole gland.

In Experiment 8 we have a condition which comes within reach of that actually existing in the animal, namely a mixture of pancreatic secretion with duodenal mucosa and bile. As a consequence all three enzymes occur in perfect harmony and fully activated without causing interfering disturbances. Under such conditions, the addition of sodium chloride does not produce further increases in the diastasic activity as Experiment 9 indicates. The importance of bile and duodenal mucosa for the proper digestion of food and the serious consequences resulting from lack of either or both, are obvious.

The diastasic enzyme is very sensitive to acids and alkalies. Addition of 0.1 per cent of concentrated hydrochloric acid to the minced pancreas lowers the diastase materially, while 1.0 per cent practically destroys it. The proteolytic power is increased somewhat by this acid treatment but not nearly as pronouncedly as by duodenal mucosa. Sodium bicarbonate lowers enzymic action gradually as the acidity of the gland decreases. It requires approximately 2.5 per cent of this salt to bring the fresh gland to litmus neutrality. On the alkaline side of neutrality all three enzymes become inert. Even distilled water depresses the enzymes. 10 per cent of distilled water added to the fresh gland reduces the diastasic activity very materially and renders the proteolytic enzyme practically inert, while the lipase is but slightly affected. Here again, sodium chloride protects the diastasic enzyme to a considerable extent.

All the pancreases examined show some degree of proteolytic power and we have been unable to locate a single sample of known source which was inert in this respect. This is shown further by the self-digesting properties of the pancreas. A number of whole glands, immediately after removal from the animals, were placed on trays kept at 50°C. under ordinary atmospheric conditions. In the course of 2 hours the glands became very soft as self-digestion rapidly took place. The fat and membranes surrounding the fresh glands formed a protective coating which held the individual gland together and prevented running. The heating was continued for 15 hours. Upon examination the interiors of the glands were found to be completely digested and still moist and soft. Many clusters of amino-acid crystals were encountered throughout the mass, indicating that the digestion was carried well into the amino-acid stage. The soft mass was spread thin on trays and dried at the same temperature. The fat-free powdered sample still showed slight proteolytic and lipolytic activities while the diastasic properties were completely lost.

The addition of 25 per cent of duodenal tissue to freshly minced pancreases increases the proteolytic power approximately fourfold over that present in the original whole gland when measured on sodium caseinate by the Fuld-Gross method, and about three times when blood fibrin and Sørensen's formol titration are employed. This discrepancy is readily accounted for when it is considered that we are dealing with two distinctly different proteins and measuring different stages of hydrolysis.

Preliminary experiments not included in the present tabulation were conducted to determine the amount of fresh duodenal tissue necessary to activate the trypsin to a maximum. Admixtures of 5, 10, 15, 20, and 25 per cent of duodenums to the pancreases were tried. While these mixtures showed increasing activity of the proteolytic enzyme they also furnished evidence of inversely proportioned decreases in diastasic and lipolytic strength.

By referring to the tabulated results it will be seen that the depressing action of duodenal mucosa on the other two enzymes is very marked in the absence of bile. The presence of bile protects the diastase and renders the lipase immune to tryptic destruction. The duodenal tissue as obtained from the healthy living animals seems to be fully sensitized for tryptic activation purposes and it

has been our experience that the addition of either hydrochloric acid or sodium carbonate or bicarbonate does not stimulate but rather tends to lower its efficiency. The activation takes place on the acid side of neutrality since both the pancreas and duodenal tissue are acid in reaction.

In another series of preliminary experiments not tabulated here it was found that small amounts (1 per cent) of the powdered 75:25 pancreas and duodenum mixture, when added to freshly minced hog pancreases, activate the proteolytic enzyme quite satisfactorily. If these mixtures, however, are employed in the same proportions for activation of new lots of glands, the results are much lower and it becomes necessary to use a higher percentage to produce the same effect. This shows plainly that tryptic activation is due to some substance present in the duodenum, with a specific but limited power of activation, and not to the activated proteolytic enzyme acting as a catalyzer. These samples also showed that the diastasic enzyme is but slightly affected by the actual tryptic activation process while the lipolytic activity is depressed materially. That it requires 25 per cent by weight of fresh duodenal tissue and only 1 per cent of the dry fat-free pancreas and duodenum mixture to produce the same tryptic activity, is readily conceivable when the general retarding nature of moist mucus is considered.

The maximum digestive power of the proteolytic enzyme is not reached at body temperature. This applies to the enzyme as it occurs in the pancreas as well as after it has been reinforced with duodenal mucosa. Fibrin digestion experiments were carried out as outlined above on representative and identical samples at 40 and also at 50°C. The six selected samples titrated 7.1, 7.2, 14.7, 16.4, 16.8, and 21.7 cc. of 0.25 N NaOH after digestion at 40°C. and 14.6, 16.0, 28.2, 31.9, 32.8, and 45.8 cc. of 0.25 N NaOH at 50°C. An actual increase of 100 per cent in the amino-acid nitrogen is obtained by raising the digestion temperature from 40 to 50°C. The importance of accurate control of time and temperature factors becomes very apparent when these results are borne in mind.

When liquid bile is mixed with freshly minced pancreases, the power of the lipolytic enzyme is almost doubled. Besides this activation property, the function of the bile seems to be that of protecting the fat-splitting process from tryptic interference. In

4. Minced hog pancreases, 90.0 per cent. Liquid hog bile, 10.0 " "	Feb. 19 March 1	50 50	63.3 60.0	17.0 22.4	19.7 17.6	1:51 1:61	1:15 1:16	7.4 7.4	45.3 45.0
Average.		100	61.7	19.7	18.6	1:56	1:15	7.4	45.2
4a. Minced hog pancreases, 90.0 per cent. Distilled water, 10.0 " "	March 4	50	66.0	16.1	17.9	1:25	1:0.5	2.4	28.5
5. Minced hog pancreases, 89.0 per cent. Liquid hog bile, 10.0 " " Sodium chloride, 1.0 " "	Feb. 19 March 4 April 1	50 50 50	62.5 63.0 59.0	16.4 16.9 24.2	21.1 20.1 16.8	1:105 1:86 1:105	1:14 1:17 1:17	7.6 7.1 7.4	44.9 41.2 41.0
Average.		150	61.5	19.2	19.3	1:99	1:16	7.4	42.4
5a. Minced hog pancreases, 89.0 per cent. Distilled water, 10.0 " " Sodium chloride, 1.0 " "	March 4	50	66.0	15.8	18.2	1:59	1:2	3.1	29.1
6. Minced hog pancreases, 75.0 per cent. " " duodenums, 25.0 " "	Feb. 4 " 19	60 30 50 30	65.0 65.0 65.0	15.7 15.0 15.0	19.3 20.0 20.0	1:35 1:24	1:100 1:117	16.6 17.4	7.8 12.5
Average.		110	65.0	15.3	19.7	1:30	1:109	17.0	10.2
7. Minced hog pancreases, 74.0 per cent. " " duodenums, 25.0 " " Sodium chloride, 1.0 " "	Feb. 4 " 19	60 50	63.5 64.5	15.6 14.4	20.9 21.1	1:57 1:53	1:89 1:111	16.8 16.8	8.8 12.5
Average.		110	64.0	15.0	21.0	1:55	1:100	16.8	10.6

combination with duodenal mucosa, bile prevents the deterioration of the sensitive diastasic enzyme. This action seems to be due principally to the sodium chloride present in the bile.

The chlorides present in hog bladder bile vary somewhat, due to local conditions and kind of food ingested. Since sodium salts predominate in bile most of the chlorine is present as sodium chloride. We found a minimum of 0.17 per cent and a maximum of 0.22 per cent of chlorine, equivalent to 0.28 per cent and 0.37 per cent of sodium chloride respectively, in the various samples of liquid bile employed in these experiments.

We are greatly indebted to Mr. Paul Rudnick, Chief Chemist of Armour & Company, for valuable assistance and courtesies which made this investigation possible. We also wish to express our thanks to Mr. Joseph Brunn for help in collecting the material and preparing the many samples.

SUMMARY.

In conclusion we wish to state that while the basic facts concerning the various enzymes of the pancreas, set forth here, are not materially new, they present, however, some points of interest.

1. The data obtained on the whole pancreas represent as nearly as possible the actual conditions existing in the living animal. The disturbance of glandular equilibrium necessarily resulting from mincing and mixing with consequent liberation of the cell contents, autolysis, etc., has been practically avoided, or at least materially minimized, by drying the entire gland *in vacuo*.

2. The normal pancreas as removed from the animal is of distinctly acid reaction and possesses high diastasic, considerable lipolytic, and some proteolytic activity. The diastasic enzyme is present in fully activated form while the strength of the lipolytic enzyme is almost doubled by addition of the bile. The proteolytic enzyme is activated to several times its original strength by duodenal mucosa.

3. Bile was found to be void of any measurable enzyme action. Besides its well known function of augmenting the activity of the lipolytic enzyme, it seems to protect the fat-splitting process against tryptic interference. Combined with duodenal mucosa, it stabilizes the otherwise sensitive diastasic enzyme.

4. It is possible to produce and maintain maximum activity of the three enzymes in the removed pancreas, without causing interfering disturbances, by the addition of adequate amounts of bile and duodenal mucosa.

5. Certain characteristic behaviors and interrelations of the enzymes in given samples are brought out.

STUDIES OF THE CONCENTRATION OF CATALASE IN URINE, CHYME, AND FECES.

By A. V. S. NORGAARD.

(From the Medical Clinic of the University of Copenhagen, Copenhagen, Denmark.)

(Received for publication, May 21, 1919.)

Estimation of Catalase.

When a solution of catalase is added to a solution of hydrogen peroxide, decomposition of the hydrogen peroxide takes place if the reaction of the mixture is not too acid. By the decomposition water and molecular oxygen are produced. The volume of oxygen is a measure of the extent of the decomposition. Coincidentally with this process a destruction of the catalase takes place, if the hydrogen peroxide solution is not too weak. Consequently the reaction, $2 \text{H}_2\text{O}_2 = 2\text{H}_2\text{O} + \text{O}_2$, is finished within a limited time.

The pyrometer, or apparatus for measurement of the oxygen, which we have used¹ consists of a 40 to 60 cm. glass tube, which is bent as shown in Fig. 1—two parallel tubes connected by an M-shaped middle piece. One of the tubes, the measuring tube, is long (20 cm.) and is provided with a graduation. The other is short (5 to 8 cm.) and provided with a piece of rubber tubing about 2 cm. long (Fig. 1). If the free end of the rubber tubing is pinched together, the short limb of the pyrometer is shut, and by so doing one may adjust the column of liquid in the pyrometer. The internal diameter of the tube is 0.5 cm.

Principle of the Method.—The principle of the method is that a measured quantity of the solution of catalase is placed in the pyrometer, which is filled with H_2O_2 , and is confined in it. The catalase will decompose a certain quantity of H_2O_2 . The liberated oxygen depresses the liquid in the short limb of the pyrometer

¹The apparatus may be purchased from the Emil Greiner Co., 55 Fulton Street, New York.

and raises the column of hydrogen peroxide in the measuring tube to an extent which indicates the volume of oxygen evolved.

Determination.—From a burette or a pipette as much 3 per cent hydrogen peroxide is run into the pyrometer through the measuring tube as is necessary to fill the M-tube and the lower parts of the two vertical tubes. Then 0.2 cc. of the catalase solution is added by letting it run from a pipette through the rubber tubing down into the H_2O_2 in the short limb. The inside of the rubber tubing is washed with two to three drops of H_2O_2 , which one lets run down into the pyrometer. Then the pyrometer is placed at such an angle that the surface of the liquid in the measuring tube stands just at the height of the zero point. While the pyrometer is held in this position, a pinch-cock is placed on the free end of the rubber tubing, which is closed by this means. The apparatus is placed in the stand.

The oxygen liberated is received partly in the short limb, partly in the nearer curve; if much oxygen is developed some will also pass over into the other curve. The displacement of the liquid in the measuring tube indicates the volume of liberated oxygen. The process is finished after 3 to 24 hours, most quickly when smaller concentrations of catalase are present.

Hydrogen Peroxide Unit of Measurement (HPU).—As unit of measurement is used the proportion between the volume of oxygen developed and the volume of catalase solution which caused it.

0.2 cc. of a liquid in a tube with an internal diameter of 0.505 cm. will fill a space 1 cm. long. Therefore if one is measuring the decomposition, which is brought about by 0.2 cc. of a solution of catalase, and finds that the surface of the liquid in the measuring tube is raised n cm., there will be developed n times as much oxygen as the volume of added solution of catalase run in. In this paper the definition of the number of units of the catalase solution therefore is n . This unit is called the "hydrogen peroxide unit" (HPU).

Sources of Error.—The reaction between catalase and H_2O_2 ought to take place in a moderately acid medium. The presence of alkalies will cause non-specific decompositions. Mineral acids prevent or diminish the process, even when they are very much diluted. It is necessary to be sure that the 3 per cent solution of

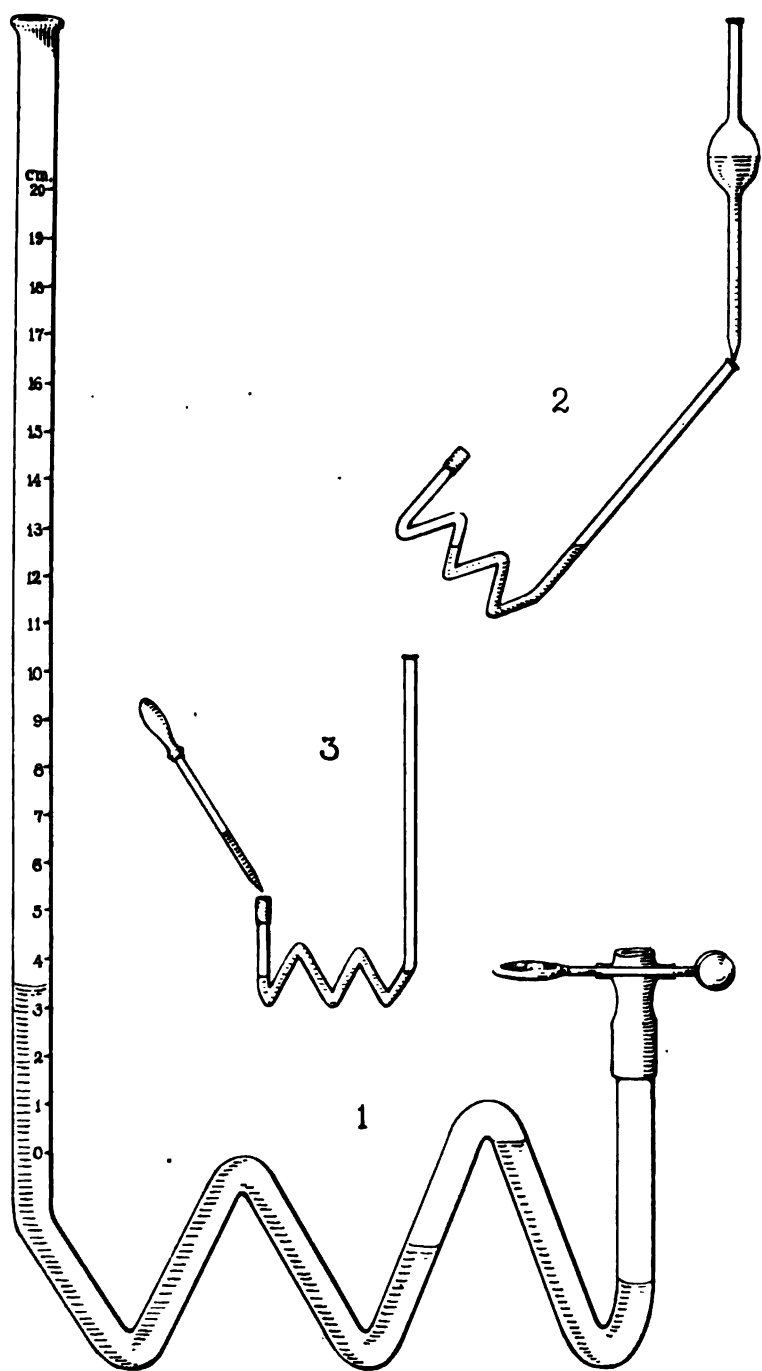


FIG. 1. 1. Pyrometer in action. 2. Addition of peroxide. 3. Addition of catalase solution.

hydrogen peroxide is neither too acid nor is decomposed spontaneously by standing.

For doing away with an alkaline reaction I add small amounts of boric acid crystals to the solution of catalase.

The determination is carried out at room temperature, but the reacting solution must be protected from direct sunlight. It is emptied by pouring the contents out through the short limb, which by that means is rinsed. Further cleaning is seldom necessary.

Estimation of Catalase in Urine.

Demonstration of pus in urine is possible by (1) examination with an alkali (Donner), (2) microscopy, and (3) reaction with hydrogen peroxide. The first of these methods is not suitable for quantitative use, the second is troublesome and cannot be used when the pus corpuscles are disintegrated, while by the third method quantitative determination is easily performed suitable for clinical use.

Determination of Catalase in Urine.—The urine is acidulated as soon as possible with boric acid crystals, as it is important that the free ammonia of the urine be neutralized. After that 0.2 cc. of urine is introduced into the pyometer, which is filled in advance with hydrogen peroxide through the long limb. The urine is added through the short limb, which is washed with three drops of hydrogen peroxide; the surface of the liquid in the measuring tube is brought to the zero point of the graduation; the pinch-cock is put on and the pyometer is placed in a shady place for a definite period.

Acidity of the Urine.—The catalase reaction takes place most quickly in a neutral or a very moderately acid medium. With more acid reaction the process is retarded and it is prevented more or less completely by a strongly acid reaction. The highly dissociated acids (HCl, H_2SO_4 , etc.) have an inhibitive influence; while the slightly dissociated acids have a smaller influence on the process, because they do not remove the hydrogen ion concentration so far from the neutral point. Such acids are, for instance, uric acid and boric acid. The latter even in half saturated solution does not affect the influence of the catalase appreciably. If the medium is on the alkaline side of the neutral

point, non-specific decomposition of hydrogen peroxide may occur, because small amounts of alkali (ammonia, carbonate) will decompose H_2O_2 . If the mixture is very strongly alkaline, the process is totally prevented, even if catalase is present. Before the urine is used for examination in the pyrometer, one must therefore test the reaction to litmus paper. If it is not acid, it should be acidulated with boric acid crystals.

Catalase in Urine.—Catalase in urine may originate from pus cells, from red blood corpuscles, from epithelial cells, from casts of the urinary tubules, from bacteria, and from alkaline products. The catalase content of casts of the urinary tubules and of the epithelial cells is quantitatively of secondary importance. *Bacillus coli* is very deficient in catalase, while the urea-decomposing bacteria produce substances that give a non-specific decomposition which may be prevented by admixture of boric acid. The remaining sources of catalase are then red and white blood corpuscles. A distinction between the catalase which originates in the red and that which originates in the white blood corpuscles is impossible. On the average a white blood corpuscle contains twice as much catalase as a red one. If red and white blood corpuscles are found together, the estimation described indicates the sum of the catalase from the leucocytes and that from the erythrocytes.

Effect of Urinary Sediments.—If the urine sample is quite fresh, the catalase content may be greater in the sediment than in the supernatant urine. If the urine has been standing for some time, however, the catalase will have left the cells, and in that case one gets the same values, whether one measures the catalase in the filtered urine or in the sediment. So, as a rule, filtration may be regarded as unnecessary.

As to the crystalline deposit, urates and oxalates are indifferent, while uric acid crystals can in certain cases produce a non-specific decomposition of the hydrogen peroxide. Phosphates seem to be indifferent, while ammonium salts and carbonates give a non-specific decomposition which may be obviated with the use of boric acid.

The volume of oxygen liberated is observed after the reaction has ceased. With small amounts of catalase the process is ended after 3 hours; with greater quantities it takes longer, but never

more than 24 hours. Consequently one may always note the results after 24 hours. The specific decomposition has then reached its maximum. If one waits longer, one sees a continued evolution of oxygen. This is corrected for by a control test in which the pyrometer is filled with hydrogen peroxide but the solution of catalase is not added. The elevation in the control test will amount to about 0.1 (= 1 mm.) in 24 hours at usual room temperature and with the solution of hydrogen peroxide (Oxydol, Petri) that I have used.

The results depend on the quantity of pus and blood that are contained in the urine. Where the catalase is caused exclusively or chiefly by pus, one will, as a rule, find HPU values from 0.1 to 20, most frequently fluctuating between 1 and 10. According to catalase content, one may divide pyuria in groups.

In all of the more than 1,000 cases examined, it has been shown that excellent agreement exists between the catalase content and the pus content estimated in other ways (microscopy, Donner's test).

Clinical Results.—1. Normal urine contains only inconsiderable traces of catalase corresponding to the few epithelial cells and leucocytes which are normally found.

2. The catalase values in urine from patients suffering from pyelitis give a practical measure of the amount of cells in the urine sample.

3. By the proposed method of estimation of catalase one can divide pyurias into limited groups according to whether the catalase content is small or great, and it is easy to distinguish among (a) acute pyurias, where catalase is only temporarily present, (b) chronic pyurias, where the catalase numbers fluctuate about the same value for a longer time before they slowly approach zero, and (c) intermittent pyurias where the catalase numbers fluctuate widely between low and high values.

4. The method permits a reliable estimate of the effect of therapeutic measures on the condition.

Fig. 2 shows different types of pyuria.

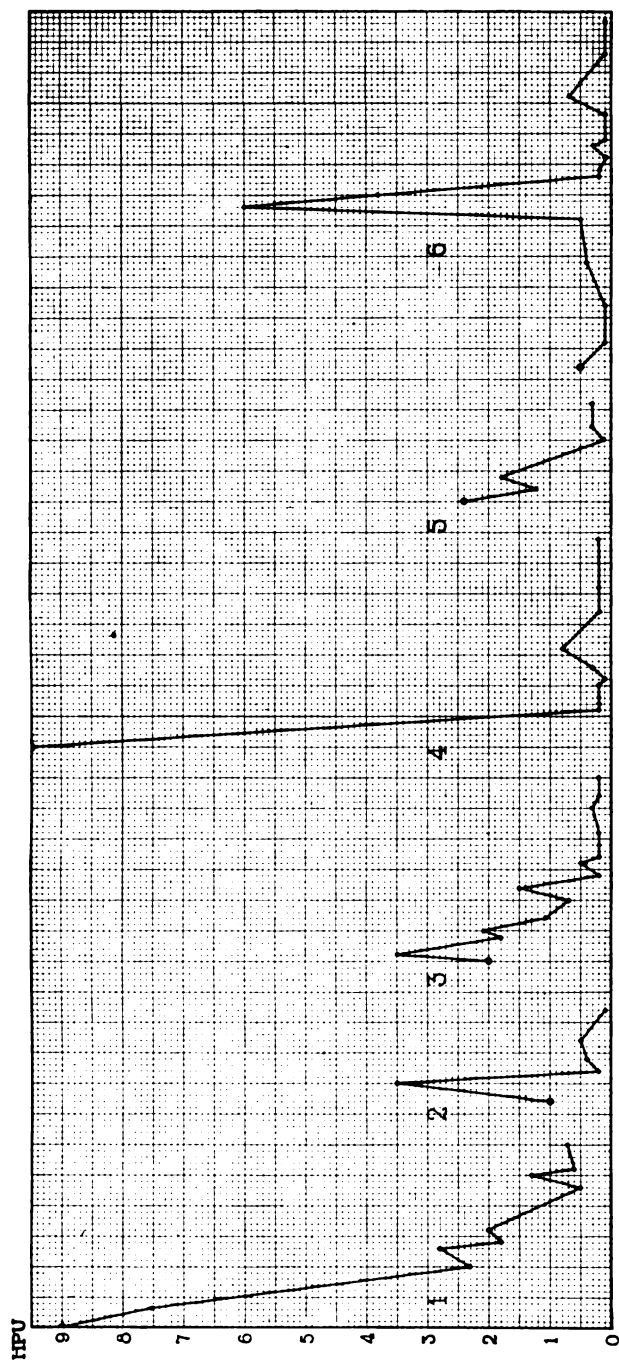


FIG. 2.

Estimation of Catalase in Chyme.

In a great number of cases I have examined the concentration of catalase in chyme and gastric juice with the technique described above. For the sake of comparison and for practical reasons I have chosen the usual clinical test meals and fasting gastric juice.

Determination.—In all cases the filtrate from the test meal has been used for examination. Usually 0.2 cc. of filtrate has been added to the hydrogen peroxide in the pyometer, and only where the decomposition was so violent that the catalase value could not be estimated in the pyometer (HPU values over 15 to 20), fractions of 0.2, namely 0.05 or 0.02 cc., have been used, the fractional proportion being taken into consideration by the calculation of the HPU value of the filtrate. If, for instance, the value 10.2 was found with 0.05 cc. of filtrate, the HPU number in the filtrate is four times as great, or 40.8.

When the filtrate from the test meal possessed a reaction near the neutral point, a couple of boric acid crystals have been added.

Catalase in the Food.—The test meals are very poor in catalase, because this enzyme is destroyed in the preparation of the food. If the filtrate from the chyme gives catalase reaction, this must be caused by the addition of catalase from the organism, and may proceed from the cavity of the mouth, from the esophagus, or from the stomach.

Catalase from the Mouth.—This originates chiefly from the salivary corpuscles, but also in some degree from the epithelial cells of the mouth. In the examination of specimens of saliva from healthy and ill people I have found HPU numbers, which have varied between 0.8 and 14.8, an average of 5.7. In a case of stomatitis much higher values were noted. The samples of saliva must be acidulated with boric acid before the estimation.

Catalase of the Secretions of the Esophagus.—This does not seem to differ from that of the saliva.

Catalase of the Chyme.—This may originate from pus cells, red blood corpuscles, or the epithelial cells of the stomach.

Clinical Results.—1. In healthy persons the filtrate from a test meal is found free from catalase.

2. In case of hypersecretion or hyperacidity the same is true.

The value of the catalase concentration is directly dependent on the presence of free hydrochloric acid (reaction by Boas' test). In the examination of 800 acid test meals catalase was never found in the filtrate when the test meal contained free hydrochloric acid.

3. One finds a positive catalase reaction in a number of cases of anacidity (achylia gastrica and cancer ventriculi).

4. In simple chronic gastritis (achylia gastrica) a positive catalase reaction in the filtrate has been found in a great number of cases. The HPU values are dependent on the acidity. In single cases, as well as in different ones, the rule is that the HPU values are inversely proportional to the total acidity.

5. 60 cases of gastritis of very different origin have been examined. None of these groups differs so much from the others that the supposition of a particular form of inflammation thereby is affirmed. The HPU values varied between 0 and 20. Usually the value was lower than 6.0.

6. Leucemia (lymphatica and myeloidea) does not cause a particular increase of the catalase concentration in the filtrates. In these diseases a secretion of leucocytes from the mucosa of the stomach has therefore hardly taken place.

7. Cancer cardiae does not differ from the simple chronic gastritis in regard to the concentration of catalase in the chyme.

8. In cancer corporis ventriculi a considerable increase of the catalase concentration in the filtrate is found, even where the test meal is not mixed with blood. The catalase is supposed to proceed from the disintegration of cells. The HPU values varied between 20 and 84.

9. In cases of cancer pylori without stagnation the catalase concentrations of the filtrates were found to be the same as by the simple gastritis.

10. Where cancer pylori was followed by stagnation (lactic acid), the catalase reaction did not take place.

11. The catalase content of chyme varies inversely with the ability of the stomach to produce hydrochloric acid, and directly with the necrosis and disintegration of cells.

12. *Negative catalase reaction* in the filtrates is therefore found partly under normal circumstances, partly in hyperacidity, and partly in some cases of simple gastritis and in cancer pylori with stagnation in the stomach.

Moderate catalase reaction is found in simple gastritis, in cancer cardiae, and in cancer pylori without stagnation (HPU values 0 to 20).

Violent catalase reaction is found only in cancer corporis ventriculi (HPU values over 20; empirically the limit is found to be 20 in the cases examined).

Estimation of Catalase in Feces.

The estimation of pus in feces is rather difficult. The microscopical examination for pus cells gives positive results only in a small number of cases, because the pus corpuscles are quickly destroyed through the action of the bacteria and the intestinal juice.

The symptoms of intestinal inflammation—pain, diarrhea, and admixture of mucus to the feces—are not quite pathognomonic. They may arise reflectorily, and they do not necessarily indicate an inflammation. It is otherwise with the proliferation of pus cells. Pus is a measure of the extent of the inflammation, and an estimation of the pus corpuscles therefore allows one to judge the extent of the inflammation. The estimation of catalase is an estimation of cells.

Determination.—The quantity of catalase is examined in an extract of the feces.

About 5 gm. of feces are weighed and mixed with three times as much half saturated boric acid solution to a homogeneous fluid. The mixture is allowed to stand 1 hour, then it is filtered; the filtration generally proceeds slowly. The catalase ought to be estimated within a half hour, as the concentration of the catalase in the filtrate diminishes on standing. For the examination one usually uses 0.2 cc. of the filtrate, but when the quantity of catalase is great, one must use smaller portions, 0.05 or 0.02 cc. In the calculation of the HPU number of the feces the used quantity of filtrate as well as the proportion 1 : 3 must be taken into consideration. The further technique is as described above. If after 24 hours with 0.02 cc. of the filtrate, the HPU number 10.2 is noted, the HPU number of the feces is $10.2 \times 10 \times 4 = 408$.

The catalase of the feces originates from the intestinal tube.

In the foregoing it was shown that the food was nearly catalase-free when it left the stomach. Where this is not the case,—in achylia gastrica—the catalase is destroyed by the enzymes of pancreas, by trypsin, and erepsin. The catalase in the feces therefore originates from the pus corpuscles, the red blood corpuscles, the epithelial cells, and the bacteria of the intestines. The estimations show that bacteria and epithelial cells are of secondary consequence. The main part of the catalase comes from the red and white blood corpuscles. Therefore with every catalase reaction a blood reaction should be performed to ascertain how much blood there is in the feces. Not all blood is of importance for the catalase reaction in feces. Blood from the food and blood which comes from the mouth, esophagus, and corpus ventriculi are of no importance for the estimation of catalase in feces, as the catalase in the blood is destroyed in the stomach; on the other hand, bleeding ulcers near the pylorus and in the intestine will increase the concentration of catalase in the feces.

Clinical Results.—1. In normal feces the average of the HPU numbers is 1.5. The lowest value found is 0.2 and the highest 3.5 in normal cases. The numbers vary most often between 0.5 and 2.5. The softer the feces, the lower is the HPU number (Fig. 3, No.1). In pathological cases the values may reach 1,000. In the clinical examination there is a great interval between the physiological and the pathological values.

2. There are some cases of gastritis (achylia gastrica) in which the HPU members lie within the same limits as the normal.

3. In bleeding gastric ulcer the blood in most cases causes an increased quantity of catalase in the feces, when the ulcer is situated at the pylorus or in the duodenum, or when a gastroenterostomia has been made. Normal numbers (0.5 to 3.5) are found when the ulcer is in the corpus ventriculi near the cardia.

4. During the treatment with milk for gastric ulcer, as well as in all other cases where the food consists of milk for a long period, a reaction (enteritis) from the intestine will occur sooner or later.

5. In bleeding gastric ulcer one may often see an increase in the HPU numbers of the feces, at first as a consequence of the hemorrhage, later as a consequence of the treatment with milk (Fig. 3, No. 2).

6. In uncomplicated constipation (intestinal stasis) the catalase concentration of the feces is decreased in the filtrate, because the catalase has been destroyed during the stay in the intestinal canal (HPU numbers 0.2 to 1.0, Fig. 3, No. 3).

7. In cases of achylia with constipation the same low numbers are found.

8. Nervous diarrhea has low HPU numbers. So it must be interpreted as an abnormal peristalsis and not as an inflammation (Fig. 3, No. 4).

9. In Graves' disease with diarrhea we find both increased peristalsis and a transudation of a secretion containing catalase.

10. Diseases of the intestines with diarrhea may be divided into two groups: (a) the diseases where the HPU numbers are not increased (nervous diarrhea, diarrhea in Graves' disease, flesh poisoning, cases of mucus and membranaceous colitis, diarrhea in influenza, and by a few cases of achylia; (b) the diseases where the HPU numbers are increased (infectious forms).

11. In intestinal fermentation acid products are found in the intestinal canal. These destroy the catalase. Consequently one finds low HPU numbers, where this disease is not connected with a secondary inflammation (Fig. 3, No. 5).

12. In several cases hemorrhages from the intestinal canal have caused high HPU numbers in the feces. Here one may distinguish between a *vascular* type (Fig. 3, No. 6), where the increase is great, but of short duration and with intercalated low or normal numbers, and a *parenchymatous* type (Fig. 3, No. 7), where the elevations are more or less high but where normal values are lacking.

13. Bleeding hemorrhoidal tumors give HPU numbers of the vascular types, cancer coli and cancer recti, of parenchymatous type.

14. The diseases of the intestine which are caused by infections have high HPU numbers. This is the case in the usual acute, infectious enteritis (Fig. 3, No. 8) and in the usual chronic colitis and enterocolitis (Fig. 3, No. 9).

15. Colitis gravis (colitis chronica suppurativa) is recognized by the microscopical finding of several pus cells in feces. The HPU numbers in these cases are very high (Fig. 3, No. 10.). The same is found in proctitis suppurativa. The fluctuations of the HPU numbers may give an idea of the prognosis.

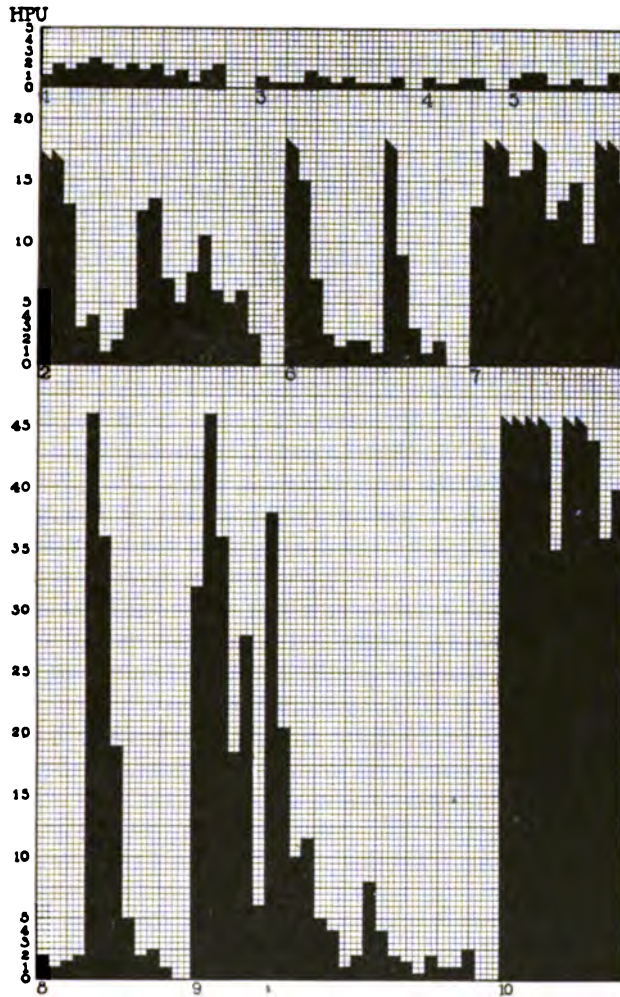


FIG. 3. 1. Normal. 2. Gastric ulcer (observe the two elevations of the curve). 3. Intestinal stasis. 4. Nervous diarrhea. 5. Intestinal fermentation. 6. Vascular hemorrhage. 7. Parenchymatous hemorrhage. 8. Enteritis acuta. 9. Colitis chronica. 10. Colitis suppurativa chronica.

16. Dysentery, typhoid fever, paradysentery, and paratyphoid fever present very high HPU numbers, as long as inflammation is present in the intestine.

17. Tubercular enteritis presents the same picture as colitis chronica and colitis gravis. The HPU numbers fall during the treatment.

18. Enteritis, which arises in connection with mercurial or arsenical treatment, shows the same type as the infectious enteritis.

19. In a large number of cases of constipation the patients examined have shown symptoms of enteritis (high HPU numbers). These are caused by irritation of the intestinal wall (colitis stercoralis).

20. In a great number of cases of achylia one finds periodically symptoms of enteritis. These periods arrive at various long intervals and are generally of short duration. These enteritides depend on the predisposition of the patients for infection, as the disinfecting HCl of the stomach is lacking.

21. The feces from patients with cancer ventriculi do not differ from those of patients with achylia in regard to the catalase concentration.

22. In most cases of intestinal fermentation one finds an enteritis which may be cured by a suitable treatment.

23. Parenteral diseases which cause abdominal pain do not increase the quantity of catalase in the feces.

24. An increase of the HPU numbers in the feces is a clinical symptom of an inflammation in the intestine and is therefore not the same as an admixture of mucus to the feces.

Modified Technique for Practical Use.

A particle of feces as large as a pea is smeared out on a clean surface (glass or porcelain). The smear is allowed to dry a little at room temperature. Then a few drops of 3 per cent hydrogen peroxide are added.

In constipation, intestinal fermentation, and non-infectious diarrhea the liberation of oxygen is minimal.

With normal feces but slight oxygen liberation takes place. In infectious diseases of the intestine or on addition of fresh blood a violent evolution of oxygen occurs. It is very violent if an abundant quantity of pus is present. In this case it is advisable to test for blood with the benzidine reaction.

CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION?*

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In modern dairy farming clover or alfalfa has become recognized as an almost indispensable roughage. The farmer has been taught that these materials are high in protein, in consequence of which they can supplement his more starchy grains and low protein silage. Therefore, the "home grown ration," consisting of the cereal grains, silage, and clover or alfalfa hay would become, in the minds of some farmers and teachers, sufficiently adequate in protein content for high milk production.

With the newer view-point of protein chemistry emphasizing the fact that the value of a protein mixture for growth or milk production will depend upon its qualitative and quantitative make-up and not merely on the quantity of the proteins ingested, it is impossible to state whether the "home grown ration" would furnish a protein supply of proper quality for high milk production without drawing on the protein tissue reserves of the animal.

The question raised can be settled only by metabolism investigations. In the present state of our knowledge it is just as probable as not that a ration made of clover hay, corn silage, and corn-meal would furnish an excellent and adequate protein mixture. The time is past for set nutritive ratios. A protein intake that will keep a cow in nitrogen equilibrium and maintain her flow of milk is all that is required and the quantity of proteins necessary to accomplish this will depend on the source of the proteins and on the quality of the amino-acids they can furnish.

In milk production we have undoubtedly followed in the past

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

a safe procedure by using a narrow nutritive ratio, which implies a liberal protein intake, for all the evidence accumulated indicates that the common sources of proteins used in feeding dairy cows,—and they have always been plant sources—will not yield a protein mixture of the highest possible production value. Our experience with a “home grown ration,” the results of which are here reported, is in harmony with this view. A protein mixture derived from clover hay, corn silage, and corn grain, oat grain, barley grain, or a mixture of the three grains will be a protein mixture of comparatively low efficiency for maintenance and milk production. It will suffice for cows of large consumption capacity and low mammary gland activity, in other words, for poor cows. But where a cow comes into lactation with a large milk flow her consumption capacity will be inadequate to furnish from such a ration sufficient protein for a positive nitrogen balance or equilibrium and the maintenance of a high milk yield. The result is a partial withdrawal of tissue nitrogen for milk production with an accompanying rapid decline in milk flow.

Undoubtedly the fact that generally in dairy practice the proteins used are of low production value and that the plane of protein intake often fed dairy cows is lower than it should be, is partly responsible for the rapid decline in milk production during the progress of lactation. Probably more cows than imagined are in negative nitrogen balance during the early period of lactation and under such conditions rapidly decline in milk flow to offset the losses sustained by autolyzing tissue.

It is undoubtedly true that some cows of strong milking tendencies and with a considerable reserve of muscular tissue will maintain for a long time a constant level of milk protein production even when in a negative nitrogen balance, but more often the tendency will be toward a condition of equilibrium.¹ That is exactly what happened in the experiments to be detailed below.

The experiments reported here involve only clover hay, corn silage, and the cereal grains. While the high protein legume seeds, such as peas and beans, are “home produced” yet their relatively low yield per acre and high market value for human food have not made them common adjuncts to ration construction on dairy farms.

¹ See records of Animals 1 and 2, Charts 1 and 2, *J. Biol. Chem.*, 1918, xxxv, 367.

EXPERIMENTAL.

The plan followed was to use a ration of corn silage and clover hay (medium red) to which ground oats were added in the first period of 4 weeks; ground barley in the second period of 4 weeks; ground corn in the third period of 4 weeks; and a mixture of approximately an equal part of the three grains in the fourth period of 4 weeks. Provision for a constant energy supply in the different rations was made through the use of corn-starch. The proportion of grains and starch fed to Cows 1, 2, and 4 was in conformity to a rule of animal husbandry teachers, namely, 1 pound of grain to 3 pounds of milk, while in the case of Cows 3 and 5 a departure from this rule was made. In the latter cases a larger proportion of grain was used.

Originally our plan was to use but three animals, carrying each one through four succeeding periods of 4 weeks each. This, however, could not be accomplished. Cows 1 and 3 were fairly large producers, producing 34 to 37 pounds of milk daily at the time the experiment was initiated. Although these cows were given all the ration they would consume they were in a negative nitrogen balance from the beginning of the experiment for from 5 to 6 weeks and gradually decreased their milk flow to a point where the ration consumed maintained a state of nitrogen equilibrium; this point was reached before the termination of the second feeding period. To continue these individuals during the next 4 weeks feeding period with the corn grain ration would not have answered the question for which we sought an answer, namely, could our protein mixture derived from corn grain, clover hay, and corn silage maintain a high milk production and nitrogen equilibrium? Consequently two other animals, Cows 4 and 5, were substituted at this point and continued through the corn and mixed grain periods.

Two of the animals used were pure bred Guernseys, one a grade Jersey, and two pure bred Holsteins. They were fed twice daily, the excreta were quantitatively collected and analyzed daily, while the milk was a 7 day composite sample. The composition of the rations fed is shown in Table I. A nutritive ratio of approximately 1:8.8 was obtained, with a total protein intake varying from 2.1 pounds per day for the smaller grade Jersey

TABLE I.
Composition of Rations.

Oat grain ration, fed daily.									
	Animal 1.			Animal 2.			Animal 3.		
	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.
	lbs.	gm.		lbs.	gm.		lbs.	gm.	
Silage.....	25.5	37.1	4.22	25.5	37.1	4.22	25.2	44.0	4.15
Clover Hay..	10.2	83.3	3.53	10.2	83.3	3.53	7.2	56.7	2.49
Grain.....	8.1	56.7	5.35	8.1	56.7	5.35	9.9	76.4	6.55
Starch.....	2.1	—	2.10	2.1	—	2.10	3.6	—	3.60
Total.....	45.9	177.1	15.2	45.9	177.1	15.2	45.9	177.1	16.79
Barley grain ration, fed daily.									
	Animal 1.			Animal 2.			Animal 3.		
	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.
	lbs.	gm.		lbs.	gm.		lbs.	gm.	
Silage.....	23.7	35.9	3.95	25.5	37.1	4.2	23.7	35.9	3.95
Clover hay...	9.5	74.9	3.29	10.2	83.3	3.65	6.7	54.4	2.32
Grain.....	6.2	54.2	5.16	6.7	56.7	5.31	7.6	64.5	6.03
Starch.....	1.9	—	1.90	2.0	—	2.00	3.4	—	3.40
Total.....	41.3	165.0	14.3	44.4	177.1	15.1	41.4	154.8	15.7
Corn grain ration, fed daily.									
	Animal 4.			Animal 2.			Animal 5.		
	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.
	lbs.	gm.		lbs.	gm.		lbs.	gm.	
Silage.....	26.6	43.1	4.4	25.5	37.1	4.3	28.9	45.4	4.8
Clover hay...	10.6	83.5	3.6	10.2	83.3	3.5	8.2	64.4	2.8
Grain.....	8.7	58.7	8.1	8.3	56.7	7.3	11.7	80.5	10.2
Starch.....	—	—	—	—	—	—	1.2	—	1.2
Total.....	45.9	185.3	16.1	44.0	177.1	15.1	50.0	190.3	19.0
Mixed grain ration, fed daily.									
	Animal 4.			Animal 2.			Animal 5.		
	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.	Weight.	Total N.	Therma.
	lbs.	gm.		lbs.	gm.		lbs.	gm.	
Silage.....	26.6	43.1	4.4	25.5	37.1	4.3	28.9	45.4	4.8
Clover hay...	10.6	83.5	3.6	10.2	83.3	3.5	8.2	64.4	2.8
Oats.....	2.7	55.0	6.2	2.6	53.9	5.8	3.6	75.4	9.3
Barley.....	2.2			2.1			3.0		
Corn.....	2.9			2.8			3.9		
Starch.....	1.4	—	1.4	1.4	—	1.4	2.5	—	2.5
Total.....	46.4	181.6	15.6	44.6	174.3	15.0	50.1	185.2	18.4

(No. 3) to 2.6 pounds for the larger Holstein animals (Nos. 4 and 5). In some instances not all the ration allowed was consumed. The nitrogen content of these residues was deducted from the total nitrogen fed. In Tables II, III, and IV are given the records

TABLE II.
Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 1.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 3-9.....	1,239.7	544.3	239.3	516.9	- 60.8	227.8
" 10-16.....	1,239.7	547.9	211.9	510.2	- 30.3	230.6
" 17-23.....	1,117.1	538.3	228.3	483.0	-132.5	226.1
" 24-30.....	1,013.6	488.0	183.0	439.4	- 96.8	208.8
Barley grain period, Animal 1.						
Dec. 31-Jan. 6.....	1,150.0	518.6	209.8	439.1	- 17.5	204.6
Jan. 7-13.....	1,155.0	527.5	197.9	404.5	+ 25.1	180.7
" 14-20.....	1,155.0	563.6	205.3	390.5	- 4.4	187.8
" 21-27.....	1,155.0	539.7	211.8	388.9	+ 14.6	171.8
Corn grain period, Animal 4.						
Jan. 28-Feb. 3.....	1,321.4	628.4	391.0	429.8	-127.8	210.5
Feb. 4-10.....	1,297.1	631.7	360.1	408.7	-103.4	191.3
" 11-17.....	1,297.1	617.4	366.4	369.4	- 46.1	178.0
" 18-24.....	1,297.1	668.7	402.4	358.7	-132.7	158.6
Mixed grain period, Animal 4.						
Feb. 25-Mar. 3.....	1,271.2	613.2	341.8	336.2	- 20.0	157.6
Mar. 4-10.....	1,271.2	608.2	310.9	342.3	+ 9.8	136.4
" 11-17.....	1,271.2	581.7	302.5	339.1	+ 76.9	133.5
" 18-25.....	1,271.2	600.2	310.5	332.4	+ 28.1	132.9

of nitrogen balance. These figures represent the intake and outgo for a 7 day period. Charts 1 to 5 graphically illustrate the nitrogen metabolism.

A study of the data shows that Cows 1 and 3 were in a decidedly negative nitrogen balance on the oat grain ration and passed to

a status of nitrogen equilibrium during the feeding of the barley ration. This is not to be interpreted as indicating a better protein mixture with the barley grain ration, but as due to a decreased production of milk nitrogen during the progress of the experi-

TABLE III.
Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 2.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 3-9.....	1,239.7	546.1	297.6	405.9	- 9.9	167.6
" 10-16.....	1,239.7	536.7	307.8	409.8	-14.6	169.7
" 17-23.....	1,239.7	564.7	289.6	395.7	-10.3	167.4
" 24-30.....	1,239.7	541.3	300.2	376.9	+19.3	165.2
Barley grain period, Animal 2.						
Dec. 31-Jan. 6.....	1,239.7	580.2	264.4	356.0	+39.7	154.7
Jan. 7-13.....	1,239.7	525.4	264.4	348.4	+99.5	149.5
" 14-20.....	1,239.7	550.0	286.5	317.0	+76.2	147.9
" 21-27.....	1,239.7	534.7	290.1	324.8	+90.1	139.0
Corn grain period, Animal 2.						
Jan. 28-Feb. 3.....	1,239.7	555.4	297.7	328.4	+58.2	141.0
" 4-10.....	1,239.7	593.4	325.7	362.8	-52.2	150.8
" 11-17.....	1,239.7	575.8	304.1	367.4	- 7.6	147.4
" 18-24.....	1,239.7	565.2	334.9	363.1	-23.5	140.0
Mixed grain period, Animal 2.						
Feb. 25-Mar. 3.....	1,220.1	566.8	332.5	354.7	-33.9	138.6
" 4-10.....	1,220.1	577.0	301.3	337.5	+ 4.3	139.9
" 11-17.....	1,220.1	602.5	303.0	342.0	-27.4	141.3
" 18-25.....	1,220.1	580.5	305.8	335.5	- 1.7	140.7

ment, with less drain on the fragments of tissue autolysis. The initial daily milk production of these two animals was well over 30 pounds and from the data it is clearly evident that such a ration will contain too poor a protein mixture for the production of so large a quantity of milk proteins without a considerable

drain on the animal's tissues. Animal 1 during the 8 weeks of feeding the oat and barley grain rations decreased the amount of nitrogen secreted in the milk per week by over 100 gm.; Animal 3 likewise decreased the milk nitrogen produced in a week by

TABLE IV.
Record of Nitrogen Balance, Milk Production, Etc.

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Oat grain period, Animal 3.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 3-9	1,200.0	497.2	206.0	542.7	- 45.9	247.9
" 10-16	1,164.7	544.9	211.8	514.4	- 106.4	228.6
" 17-23	1,039.2	496.4	168.2	457.1	- 82.5	229.4
" 24-30	984.2	435.5	168.1	435.9	- 55.3	205.0
Barley grain period, Animal 3.						
Dec. 31-Jan. 6	965.2	453.0	157.8	398.8	- 44.4	195.4
Jan. 7-13	999.6	468.7	135.9	402.9	- 7.9	184.8
" 14-20	1,002.0	470.0	142.1	377.0	+ 12.9	184.4
" 21-27	978.0	446.9	139.7	380.3	+ 12.1	168.5
Corn grain ration, Animal 5.						
Jan. 28-Feb. 3	1,296.4	685.4	266.0	420.6	- 75.6	198.9
Feb. 4-10	1,332.1	723.8	261.8	405.6	- 59.1	184.8
" 11-17	1,332.1	725.7	265.7	386.3	- 45.7	187.4
" 18-24	1,332.1	708.8	294.5	375.2	- 46.4	166.6
Mixed grain period, Animal 5.						
Feb. 25-Mar. 3	1,296.4	662.8	260.9	371.1	+ 1.6	165.7
Mar. 4-10	1,296.4	708.8	203.6	371.2	+ 7.2	165.7
" 11-17	1,296.4	714.1	216.6	370.9	- 5.2	163.5
" 18-25	1,296.4	710.1	218.8	365.4	+ 2.1	163.5

150 gm. The volume of milk produced by No. 1 on December 3, when the experiment was started, was 33.8 pounds, but 8 weeks later, or on February 3, it was but 23.7 pounds. No. 3 produced on December 3, 36.9 pounds of milk, but on February 3, or 8 weeks later, she produced but 25.9 pounds.

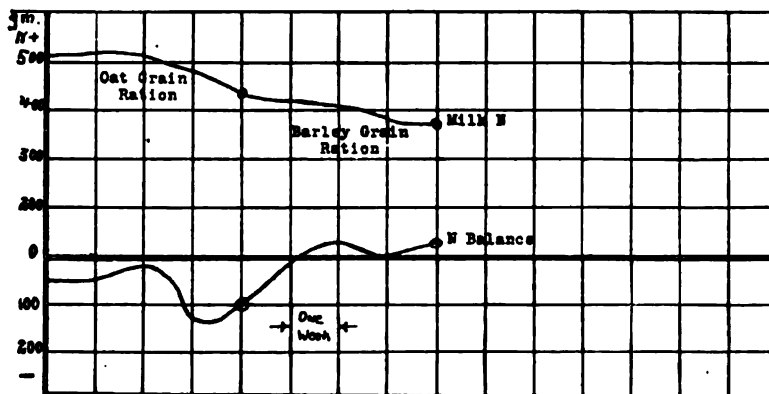


CHART 1. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

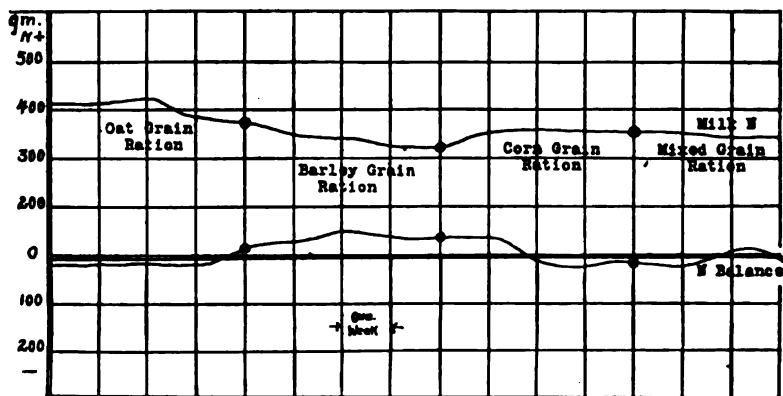


CHART 2. Showing nitrogen balances and milk nitrogen produced. This animal had a large consumption capacity and practically maintained her nitrogen equilibrium from the beginning of the experiment. The result was that over a period of 16 weeks the gradual decrease in weekly milk nitrogen elaborated was not large.

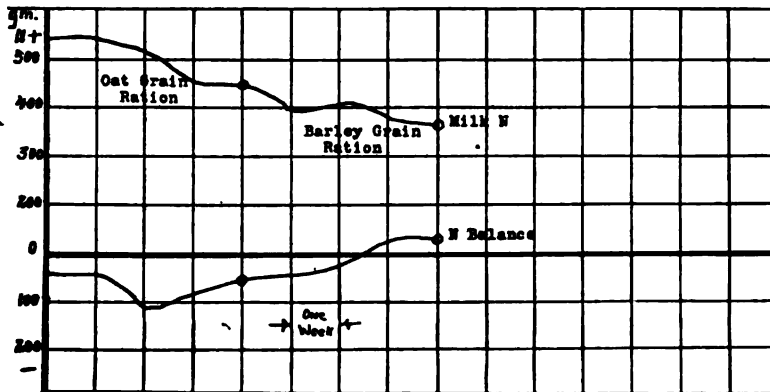


CHART 3. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

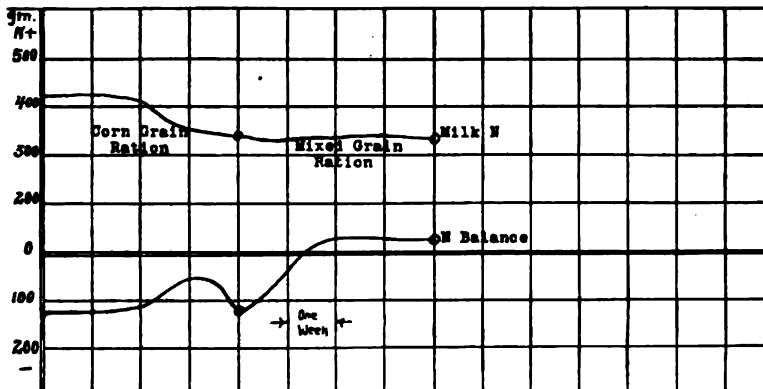


CHART 4. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

Animal 2 had a large capacity for feed, but a much lower milk-producing capacity than the other two animals, Nos. 1 and 3. The result was that she was able to maintain herself in nitrogen equilibrium with the amount and quality of the nitrogen provided by a ration of corn silage, clover hay, and the cereal grain, or a cereal grain mixture. She was continued under observation for the entire 16 weeks and during that time decreased the milk nitrogen produced per week by but 60 to 70 gm. The volume of milk also was remarkably maintained, starting with a production on December 3 of 23.7 pounds and completing the record

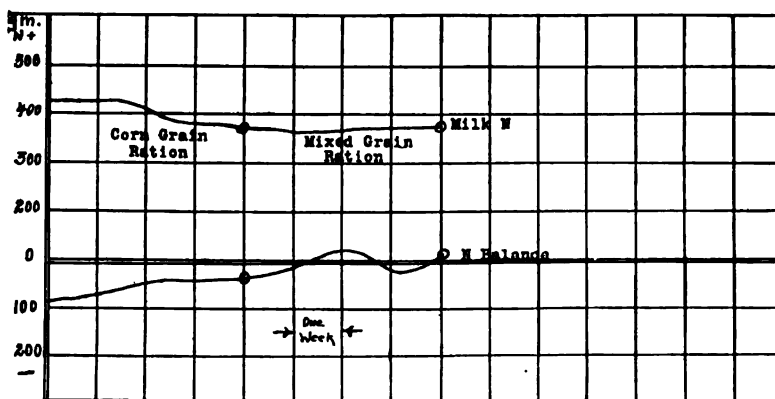


CHART 5. Showing nitrogen balances and milk nitrogen produced. Note the more rapid decline in milk nitrogen production during the period of negative nitrogen balance.

on March 17 with 20.1 pounds. This animal's record is an excellent demonstration of the fact that only moderate milk production is possible from the common "home grown" products used in these experiments.

In Table V sufficient data are presented to support the conclusion that the essential change incident to a protein intake insufficient in quantity or quality for nitrogen equilibrium in a milking animal is a rapid decrease in milk flow and not a change in the percentage composition of the milk produced.

Animal 4 (see Table II) starting with all she could consume of the corn grain ration and with a fairly high milk production

(33.3 pounds of milk per day) was immediately plunged into a negative nitrogen balance. The sequel to this condition was a rapid decrease in milk flow. Apparently the corn grain ration gave no better protein mixture than did the oat grain or barley grain ration. This animal reached nitrogen equilibrium in the mixed grain ration (oat-barley-corn mixture), but only after a decrease of 90 gm. per week in milk nitrogen elaboration and a

TABLE V.

Decrease in Milk Volume with Cows in Negative Nitrogen Balance, but Maintenance of Percentage Composition of Milk.

	Animal 1.		Animal 4.	
	Dec. 16.	Jan. 13.	Feb. 10.	Mar. 10.
Total solids, per cent.....	13.57	13.52	10.96	11.90
Fat, per cent.....	4.60	4.45	2.80	3.50
Nitrogen, per cent.....	0.49	0.47	0.41	0.48
Milk daily, lbs.....	32.7	26.8	32.9	19.6
Animal 2.*				
Total solids, per cent.....	14.27	14.26	13.88	14.17
Fat, per cent.....	4.70	4.60	4.70	5.10
Nitrogen, per cent.....	0.53	0.51	0.55	0.53
Milk daily, lbs.....	24.3	21.60	22.2	21.2
Animal 3.				
Total solids, per cent.....	13.07	12.73	12.07	12.58
Fat, per cent.....	4.10	3.50	3.10	3.60
Nitrogen, per cent.....	0.46	0.47	0.45	0.49
Milk daily, lbs.....	32.6	26.0	31.3	23.0
Animal 5.				
Total solids, per cent.....	13.07	12.73	12.07	12.58
Fat, per cent.....	4.10	3.50	3.10	3.60
Nitrogen, per cent.....	0.46	0.47	0.45	0.49
Milk daily, lbs.....	32.6	26.0	31.3	23.0

* Cow 2 maintained volume of milk, but was not in negative nitrogen balance.

shrinkage in daily milk volume from 33.3 pounds per day to 19.5 pounds per day.

The behavior of Animal 5 was an exact duplication of the records of the other high producing animals with which we experimented. There was a decided negative nitrogen balance on the corn grain-corn silage-clover hay ration, with a progressive decrease in flow of milk until nitrogen equilibrium was reached. In the case of this individual there was a decrease in daily milk flow from 31.3

pounds at the beginning of the experiment to 23.6 pounds at the time that nitrogen equilibrium was reached and the weekly amount of nitrogen secreted in the milk was reduced by 50 gm. in 4 weeks.

Our data on the value of the proteins of the mixed grain ration are not so conclusive as those on the single grain mixtures. All the animals had practically reached nitrogen equilibrium when they were changed to the mixed grain ration. From available evidence on the value of a mixture of cereal grain proteins for growth^{2,3} it is altogether probable that such a mixture of grains as used here would furnish no better quality of proteins than the single grain itself. The elaboration of milk proteins is to be looked upon as a phenomenon analogous to growth. While the distribution of amino-acids in the milk proteins is different from what it is in new tissue formed in growth, yet their origin in either case is the food supply, since probably no distinctive capacity for amino-acid synthesis is inherent in the mammary gland.⁴ Further, had the amino-acid content of the mixed grain ration been of a quality superior to that of any of the single grain mixtures used, there would have been a larger storage of nitrogen following the periods of negative nitrogen balance experienced during the corn grain period. This, however, was not the case (see records of Animals 4 and 5, Tables II and IV).

The live weight of our animals gave no indication in the period of observation as to the status of their nitrogen equilibrium. Two of them, Nos. 1 and 3, lost considerable weight under conditions of a negative nitrogen balance; No. 2 which was in nitrogen equilibrium during the entire 16 weeks neither gained nor lost appreciably in weight. Nos. 4 and 5, although in negative nitrogen balance 4 to 5 weeks showed no decrease in live weight,—in fact they actually increased in weight. Records of live weight are given in Table VI.

The animals in negative nitrogen balance decreased their milk flow in the course of 5 to 6 weeks by 8 to 10 pounds. This amount of milk would have a market value of at least 50 cents. For 7 cents per day 2 pounds of any of the common plant protein con-

² McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

³ Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1919, xxxviii, 267.

⁴ Hart, E. B., Nelson, V. E., and Pitz, W., *J. Biol. Chem.*, 1918, xxxvi, 291.

centrates could have been added to the ration, and thereby raised the plane of protein intake, without the animals experiencing any such rapid shrinkage in milk flow as took place. From what we know of the physiology of milk secretion and the relation of protein to that function it would appear to be unprofitable not to supply protein in quantities sufficient to maintain nitrogen equilibrium in a milking animal.

TABLE VI.
Records of Live Weights of Animals.

Animal No.	December 2.	January 20.	March 17.
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>
1	1,024	935	
2	1,088		1,109
3	885	762	
4		1,356	1,395
5		1,135	1,200

SUMMARY.

1. In this paper data are presented which show that it is not possible to furnish dairy cows of *high milk-producing capacity* with a protein level of sufficient magnitude or quality to maintain *that capacity* from a clover-corn silage-cereal grain mixture, the latter being corn, barley, or oats alone or a mixture of the three.

2. A cow with low mammary capacity but with large food consumption can be kept in nitrogen equilibrium by such rations. One of our animals gave 22.24 pounds of milk daily for 16 weeks and maintained nitrogen equilibrium on such a ration.

3. In the present state of our knowledge the only safe procedure for the maintenance of high milk production is through the use of a high plane of protein intake which, although of low relative efficiency, should be drawn from the plant protein concentrates rather than from those of animal origin.

Further studies will be made of the possibilities of other "home grown" protein combinations. It is entirely possible that highly efficient protein mixtures used at comparatively low levels of intake may yet be found among the common plant sources.

THE USE OF TURPENTINE RESIN IN TURPENTINE AS A FOAM BREAKER.

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(Received for publication, May 23, 1919.)

Many substances have been suggested for breaking the foam which is formed by the rapid aeration of blood and other fluids. Among the best of these agents are caprylic and amyl alcohol. If only an occasional determination is made, caprylic alcohol or amyl alcohol is entirely satisfactory and the cost is negligible, but if a large amount of work is carried on for a long time the cost of material becomes of importance.

During the past year attempts have been made in our laboratory to find a cheaper substitute for amyl alcohol. Among other substitutes, turpentine was found to have distinct foam-breaking properties. As the sample tried was not quite neutral it was decided to distill it and it was then found that the distilled turpentine had lost practically all its foam-breaking properties. The real foam-breaking agent appeared to be some impurity which was contained in crude turpentine and which could be removed by distillation. The most probable impurity was resin, and we found that on adding resin to turpentine its foam-breaking power was greater than that of amyl alcohol. We now use from 1 to 2 cc. of a 20 per cent solution of resin dissolved in turpentine. This is made by breaking the resin into small pieces and dissolving it in the turpentine on a hot water bath. During the last six months we have encountered no blood which could not be aerated at the most rapid rate with this foam-breaking agent. If the solution of resin in turpentine is not neutral to alizarin, either 0.1 N sodium hydroxide or sulfuric acid is added until the solution is exactly neutral. Resin dissolved in turpentine is an efficient foam-breaker, is very easily prepared, and is much cheaper than caprylic or amyl alcohol.

THE HYDROGEN ION CONCENTRATION OF FOODS.

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(From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.)

(Received for publication, May 29, 1919.)

Owing to the fact that the vitamins of foods are more perishable in alkaline than in acid media, it was thought worth while to increase our knowledge of the hydrogen ion concentration of foods. Such determinations may also be useful in connection with a study of the hydrogen ion concentration of the contents of the intestinal tract.

Foà¹ states that the pH of ripe grape juice is 4.52, of the juice of a nearly ripe pear is 4.24, and of the milk of *Ficus elastica* is 5.7. Clark and Lubs² give a number of determinations as follows:

Substance.	Raw.	Autoclaved.
	pH	pH
Whey.....	1.64-2.56	
Vinegar.....	2.36-3.21	
Silage juice.....	3.70-3.91	
Apple ".....	3.76-5.65	3.8
Prune ".....	4.12-9.44	4.3
Beer wort.....	4.91-8.55	
Carrot juice.....	5.21-9.27	5.2
Cucumber ".....	5.08	5.1
Apple ".....	5.02	
String bean juice.....	5.23-8.63	5.2
Banana juice.....	4.62	4.6
Potato ".....	6.06-9.44	6.1
Sweet potato juice.....	5.80-8.73	
Maple syrup.....	6.75-6.8	
Beet juice.....	6.07-8.75	6.1

¹ Foà, C., *Arch. Fisiol.*, 1906, iii, 390.

² Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

These determinations were made with the hydrogen electrode but the juices were used to standardize colorimetric methods and were apparently kept for long periods and became more alkaline, and the more acid reaction probably represents the fresh juice. They quote the following values from the literature:

	pH
Muscle juice	6.8
Pancreas extract	5.6
Milk	6.6-7.6
Flour extract	6.0-6.5
Beer	3.9-4.7
Wine	2.8-3.8
Lime juice	1.7
Lemon "	2.2
Cherry "	2.5
Grapefruit "	3.0-3.3
Orange "	3.1-4.1
Rhubarb "	3.1
Strawberry "	3.4
Pineapple "	3.4-4.1
Tomato "	4.2
Plant cell sap	5.3-5.8

Our own determinations were begun as an attempt to find the cause of the deterioration of the antiscorbutic substance between the time of its formation in the sprouting of barley and through the process of making malt extracts in various ways. We found that the acidity of press juice or extracts of steeped barley or sprouting barley or any of the products investigated was as acid as pH=5.5 and was not made less acid by exposure to air, filtration through fullers' earth, boiling, or evaporation at low pressure while a stream of air passed through it. This acidity is somewhat lower than orange juice and much lower than lemon juice. It might therefore be advantageous to add acid to preserve the antiscorbutic substance. It would require too much space to give all the determinations with the many variations in mode of treatment of the product, but the following example illustrates the character of the work. Barley of 95 per cent germination capacity was malted by the drum method until the acrospire was as long as the grain, the green malt was crushed between steel rollers, and a little water was added to make a pasty mass and pressed in a Buchner press without the addition of any other substance. The pH of the press juice was 5.4 and of the same after boiling 5.45.

We extended our observations to foods in general and made a long series of determinations with the result that they were all on the acid side of neutrality no matter what the condition of freshness, mode of storage, stage in the preparation (cooking), or dilution with water (extract, soup, or pot-liquor). We had supposed that meats, at least, might turn alkaline on cooking, due to the loss of CO_2 in the bicarbonates contained in them, but apparently enough lactic acid is developed in meat to prevent this change. For example, a piece of lean rabbit was boiled until the muscle fibers began to loosen and the press juice pH was 6.25 whereas the pH of the pot-liquor or water in which the meat was boiled was 5.89. We did not succeed in getting enough press juice of raw meat without the addition of water, as the clotting of the muscle proteins causes the water to be held rather firmly, but the following example may illustrate the work we did do. A rabbit was killed and the leg muscles placed in a mixture of ice and salt until frozen. The frozen muscles were quickly sliced and placed between steel plates and suddenly subjected to a pressure of 5,000 pounds per square inch to press out the muscle plasma. This muscle plasma was mixed with a little water and placed in a canvas bag in a Buchner press, and the press juice run into the electrode, showing pH = 6.

We made comparisons of the pH of vegetables pressed fresh, or first cooked and then pressed and found slight differences in the pH. In order to determine whether this was due to the volatility of some acid or base we pressed the juice from the raw food and determined the pH, then boiled the juice and then determined the pH. The differences obtained were very slight and might be due to changes in acid-binding power of proteins on coagulation.

Table I shows the pH of fruits and vegetables in our last series of determinations. The juice was pressed out of the raw food and the pH determined, then the juice was run into a silica dish, an

TABLE I.

Substance.	Raw.	After boiling.
	pH	pH
Young carrot juice	5.85	5.80
Potato juice	5.57	
Cabbage "	5.90	5.78
Orange "	3.55	3.55
Lemon "	2.32	2.30

equal volume of distilled water added, then boiled, and the original volume restored by the addition of distilled water or further boiling, as required.

In general it may be said that the juice became slightly more acid on boiling. On the contrary, if the food was boiled before pressing it might be slightly less acid, thus raw potato gave pH=5.57 and boiled potato 5.79, raw cabbage 5.90 and boiled cabbage 6.50.

As the technique of hydrogen ion determinations of such material has been admirably described by Clark³ it is not necessary to go into details, but we do not believe all the precautions taken by Clark are necessary. We found it necessary to immerse the platinized part of the electrode completely but not to avoid foam, and after thorough shaking the potential was maintained constant for a long period after the shaking ceased. If the solution to be tested is sufficiently viscous to support a layer of the solution on the platinized electrode (as in the case of blood plasma), it is not necessary to take the reading with the electrode totally immersed. But in the case of most plant juices the film drains off the electrode and at the same time the potential gradually changes and continues to do so for hours and it is therefore impossible to decide the correct reading. We found that palladium-coated electrodes deteriorated so rapidly that they were discarded and platinized gold electrodes used exclusively. From other work, however, we believe iridium would be superior to platinum.

Certain unavoidable difficulties enter into the determination of raw juices that prevent absolute uniformity of results. The juices contain enzymes and, if not absolutely sterile, a variable quantity of adventitious enzymes is developed. This enzyme action may be readily observed in plant juices, due to the presence of oxidases that act on chromogens such as tyrosine. We did not make a complete study of changes in acidity due to autolysis or bacterial action, but observed isolated instances of such changes. For example, freshly pressed carrot juice gave pH=5.85 whereas a portion of the same juice exposed to air 20 minutes before running into the electrode gave pH=5.73. All our observations showed increase in acidity on standing, but we interpret the tables of Clark and Lubs as indicating that the juices might become alkaline, possibly due to alkaline fermentation such as occurs in urine.

³ Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

FACTORS INFLUENCING THE HYDROGEN ION CONCENTRATION OF THE ILEUM.

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The senior author determined the pH of duodenal contents from two adults (human) collected by Dr. Schneider in April, 1915, and found one to be 7.61 and the other to be 1.5, or an average of 4.55, whereas the average of fifteen samples from infants was 4.9. Long and Fenger¹ obtained an average of pH = 5.72 for adult human duodenal contents (thirteen samples). McClendon, Shedlov, and Thomson² obtained an average of pH = 6.2 for the contents of the ileum of seven pups and McClendon, Shedlov, and Karpman³ obtained an average of pH = 6.03 in eight determinations of the ileum of four adult dogs. The discrepancy between the results on human and dog intestine may be due to the fact, as shown below, that the intestine may become less acid on the way down and the human samples could be taken from the duodenum only.

Torrey⁴ has shown that lactose and dextrin when added to a basal diet for dogs cause replacement of *Bacillus coli* by *Bacillus acidophilus* and, although the above results may not show a difference in the pH of the lactose-eating puppy and adult dog, the human data indicate such a difference and we wished to test this question more thoroughly. Adult cats were fed as much lactose in milk as they would consume. The average of five determinations on the pH of the ileum was 6.01 as compared with 6.38 for six

¹Long, J. H., and Fenger, F., *J. Am. Chem. Soc.*, 1917, **xxxix**, 1278.

²McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, **xxxi**, 269.

³McClendon, J. F., Shedlov, A., and Karpman, B., *J. Biol. Chem.*, 1918, **xxxiv**, 1.

⁴Torrey, J. C., *J. Med. Research*, 1919, **xxxix**, 415.

determinations on cats fed waste food from the table. One determination on a cat to which 200 gm. of cane sugar were administered by means of the stomach tube gave pH = 6. A dog was fed 3 pounds of milk and 1 pound of cane sugar the first day, the same the second day, and 2 pounds of milk and 2 pounds of sugar the third day, at the end of which time the pH of the jejunum was 6, of the middle ileum 6, and of the lower or distal ileum 6.7.

In rabbits it was necessary to distinguish between different regions of the intestine, as shown in Table I which gives averages on different feeds.

TABLE I.
pH of Ileum of Rabbits.

Food.	Proximal.	Middle.	Distal.
Lactose and oats.....	6.5	6.6	6.8
Carrots	6.51	6.65	6.76
" and oats.....	6.91	7.32	7.66
Oats	7.12	7.34	7.51

The results may possibly show that the more soluble carbohydrate there is in the food, the more acid the intestine may become, yet the data do not warrant any positive statements on the subject. In fact, we began to suspect that the length of the ileum might be a factor, since in long ileums the acidity decreased on the way down.

If the ratio of the length of the ileum to that of the body is taken as an index of the relative length of the former we obtained an index of 8.6 for the cat and 13 for the rabbit, using the same methods of measurement. It is then necessary to test whether the relatively shorter intestine is more acid in all cases as it is in the above comparisons of carnivora and herbivora. In order to compare the same species we used suckling and adult rabbits. The index of the former was 10 and the latter 13. (In man the reverse change occurs, the index for new-born infants being 7.9 and for adults 3.8.) So the young ones would be expected to have a more acid ileum, as was actually found, the average values being 6.4 pH for the proximal and 6.61 for the distal portions in four young rabbits. Since this was even more acid than it was possible to make the ileum of adults by diet, we infer that the milk diet of

the young is not the only factor but that the shorter intestine is associated with more acid contents.

Since we did not find appreciable amounts of ammonia in alkaline intestinal contents, and the original pH of all the food tested was acid, we concluded that the variations in pH must be due to some other factor, such as the absorption of CO₂ as the food moved down the intestine. Long and Fenger¹ determined the pH and CO₂ tension of the intestinal contents of hogs, their average figures being as follows:

Part of ileum.	pH	CO ₂ tension in per cent of an atmosphere.
Proximal.....	6.7	23.6
Middle.....	6.96	13.0
Distal.....	7.1	14.0

It therefore appears that the intestinal contents become alkaline because the CO₂ is absorbed faster than it is produced. As the gastric juice mingles with pancreatic secretion in the duodenum, a

TABLE II.
pH of Rabbit's Ileum.

No.	Age.	Proxi- mal.	Middle.	Distal.	Diet.
1	4 weeks.	6.13		6.32	Nursing.
2	4 "	6.32		6.54	"
3	8 "	6.45		6.68	" and eating oats and carrots.
4	8 "	6.73		6.90	" " " " " "
5	Adult.	6.91	7.40	7.80	Oats and occasionally carrots.
6	"	6.84	7.31	7.72	" " " "
7	"	6.8	7.28	7.62	" " " "
8	"	6.93	7.48	7.89	" " " "
9	"	6.70	6.90	7.10	" " " "
10	"	7.40	7.71	8.10	" " " "
11	"	6.81	7.20	7.39	" " " "
12	"	7.50	7.70	7.92	"
13	"	7.00	7.18	7.25	"
14	"	6.97	7.14	7.37	"
15	"	6.81	6.90	7.00	"
16	"	6.62	6.89	7.20	Carrots.
17	"	6.09	6.28	6.34	"
18	"	6.65	6.78	6.94	Water only.

large amount of CO_2 is produced more of which is absorbed the longer the intestine. Bacterial action, however, would partially restore the CO_2 absorbed. (The mineral content of the food does not seem to be a factor in determining the pH since the acidity of the intestinal contents is greater on a diet of carrots that yield a basic ash than on a diet of oats that yield an acid ash.) That the contents of the ileum become less acid on the way down is conclusively shown by Table II.

EFFECT OF DIET ON THE ALKALINE RESERVE OF THE BLOOD.

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(Received for publication, May 29, 1919.)

The beneficial effects of fruits and vegetables in the diet have been recognized for a long time. Their virtue was at one time attributed to their alkaline ash by Henry C. Sherman and others. Blatherwick¹ has shown that plums, prunes, and cranberries leave acid residues in the body due to the fact that they contain benzoic acid which is not oxidized. It is true, however, that many fruits and vegetables decrease the acidity of the urine, but it has not been proven that the total advantage of them in the diet is due to this cause. In fact the more we learn about vitamins, the more their importance rises and at the same time the importance of ash constituents of food falls.

Our interest in this subject arose from the fact that the Division of Food and Nutrition of the Army paid considerable attention to the acid-base balance of diets and it seemed worth while to show whether or not it was of importance in maintaining the alkaline reserve of blood. By alkaline reserve we mean the bicarbonate concentration of the plasma on the basis of a normal solution of sodium bicarbonate.

The blood was drawn directly from a vein or artery into a tube in which enough dry potassium oxalate had been placed to make 1 mg. per cc. of blood. This was done by measuring the required quantity of 25 per cent potassium oxalate solution into the tube and evaporating it to dryness. In the case of rabbit's blood the quantity was doubled. The blood was drawn and centrifuged with practically no exposure to air or other gas. 1 cc. of plasma was placed in a rotating hydrogen electrode and titrated with 0.1 N HCl under a stream of hydrogen to neutrality (pH = 7).

¹ Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 409.

The titration electrode was an improvement on the one previously described.² It is shown in Fig. 1. The electrode vessel was made of a 50 cc. volumetric flask with the neck cut off and the base cemented to a cork pulley-wheel with sealing wax. It was supported on a frame so that the open end would fit over a short piece of tubing attached in the frame and the base was supported by the axle of the pulley. One end of the frame supported a rod holding a micro-burette. After the plasma was placed in the vessel and the latter adjusted in the frame, a plug was carefully introduced through the short section of tubing into the electrode vessel. This plug was made of a short piece of rubber tubing through which passed a bundle of tubes the whole plug being made coherent by means of sealing wax. The bundle of tubes consisted of (1) a glass tube admitting a constant stream of hydrogen, (2) the tip of the burette, (3) a minute rubber tube filled with a saturated solution of KCl and closed at the inner end with a bit of match stick, and (4) a glass tube containing a platinum wire. The glass was fused around the platinum wire at the inner end and the outer end of the wire ended in a loop to be connected with the potentiometer. The inner end of the platinum wire had been dipped in melted gold, and before each titration it was cleaned by heating in a flame and plated with iridium by electrolyzing a strong solution (about 50 per cent) of iridium chloride with a 2 volt current for a few seconds, using another platinum wire as anode.

Since an inexhaustible supply of pure hydrogen is necessary for the continued success of the method, all previous sources of hydrogen were discarded and the hydrogen generator shown in Fig. 2 was constructed. Fig. 2 is somewhat schematic and shows only one of the battery of two electrolytic cells. Each cell was made of a beaker into which was inverted a funnel raised from the bottom by means of a piece of glass rod. Two rings of No. 10 nickel wire were made a trifle smaller than the large opening of the funnel and placed, one inside and one outside the lip of the funnel and were continuous with two upright pieces of the same wire projecting out of the cell. A mixture of 30 gm. of KOH and 100 cc. of H₂O was poured into the cell until the nickel rings were covered.

² McClendon, J. F., *J. Biol. Chem.*, 1918, xxxiii, 19.

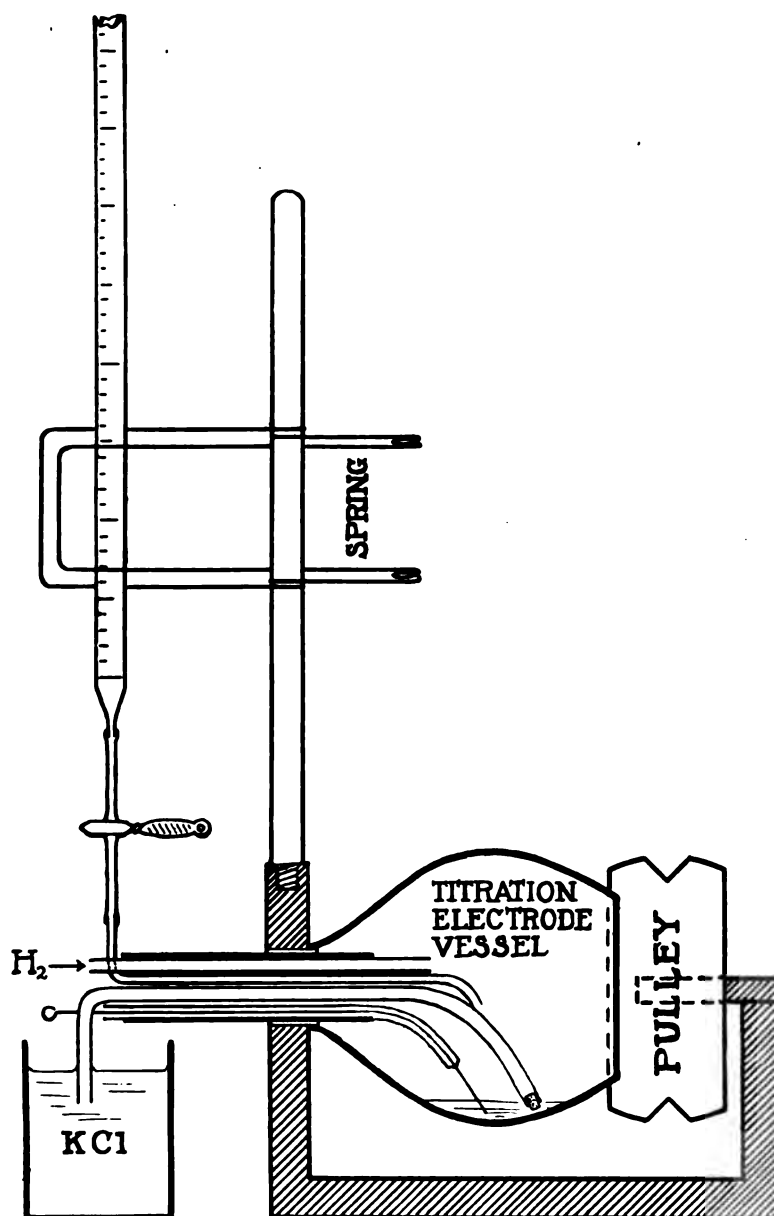


FIG. 1.

The electric current passed from one nickel ring around the lip of the funnel to the other ring with minimal heating of the electrolyte. Hydrogen was generated inside the funnel and passed up the stem and into a thick-walled rubber tube. The nickel wire

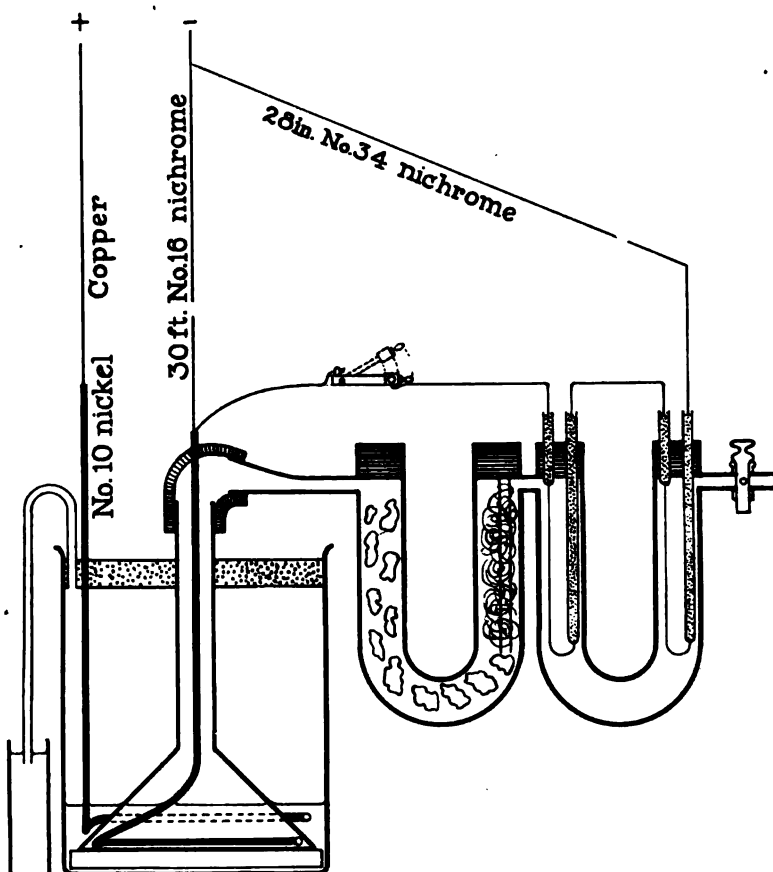


FIG. 2.

connected with the inner ring was sharpened and stuck through the rubber tube so as to connect with the source of current. The above description completes the essential parts of the generator and the additions were merely for regulating the current and pressure and removing the oxygen.

The pressure was regulated by retarding the escape of oxygen and this was done by closing the beaker with a sheet of metal covered with a thick layer of sealing wax. This cover was pierced by three openings, one for the funnel stem, one for the outer nickel wire, and one for a tube leading the oxygen to a trap filled with water to regulate the pressure.

The hydrogen passed from the cell to a U-tube filled with CaCl_2 and cotton and then to another U-tube, each arm of which contained a red-hot platinum wire 0.03 mm. in diameter to burn up the oxygen. If the gas is dried before reaching the hot wire, the water formed by combustion will not condense in the U-tube even though it is immersed in cold water to keep it cool (as we do it). The gas is finally passed through a wash bottle filled with 1 per cent NaCl to moisten it, and a spiral copper tube to bring it to the temperature of the room and then into the electrode.

The source of current was 110 volt direct current and the two cells were connected in series. The apparatus was connected to the main current by means of 30 feet of No. 16 nichrome wire stretched free in the air. Part of the current which passed the cells was then shunted off and used to heat the platinum wires. The resistance of the 6 inches of platinum wire was not enough so it was increased by means of 28 inches of No. 34 nichrome wire. When the current was turned off from the cells the platinum wires could not be heated. On first starting the apparatus with air in the U-tubes, an explosion would occur that would break the fine platinum wire, and in order to avoid this, a switch was placed in the shunt so that the air could be washed out with hydrogen before the platinum wires were heated. 20 amperes of current passed through each cell and about 5 cc. of H_2 per second were produced.

The handling of the fine platinum wire requires some care. Two glass tubes were passed through each rubber stopper of the U-tube and pieces of larger platinum wire fused in their lower ends. A little gold bead was fused on the end of each wire by touching it in a flame with a gold wire. The 0.03 mm. platinum wire was laid across the gold bead and caused to adhere by reheating it for an instant, the process repeated for the other gold bead, and then the excess of the fine platinum wire was cut off. The glass tubes were filled with mercury and copper wires carrying the current dipped in them.

In making the titrations, if enough plasma was available for triplicate determinations, the first titration was done roughly and usually overran the point of neutrality. The amount overrun was calculated from the titration curve, Fig. 3, and a second titration made by dropping in all the acid at once. Then a third titration duplicated the second. Although the titration curve in Fig. 3 is from one plasma only and may differ somewhat from other plasmas, it is very useful when applied in this way.

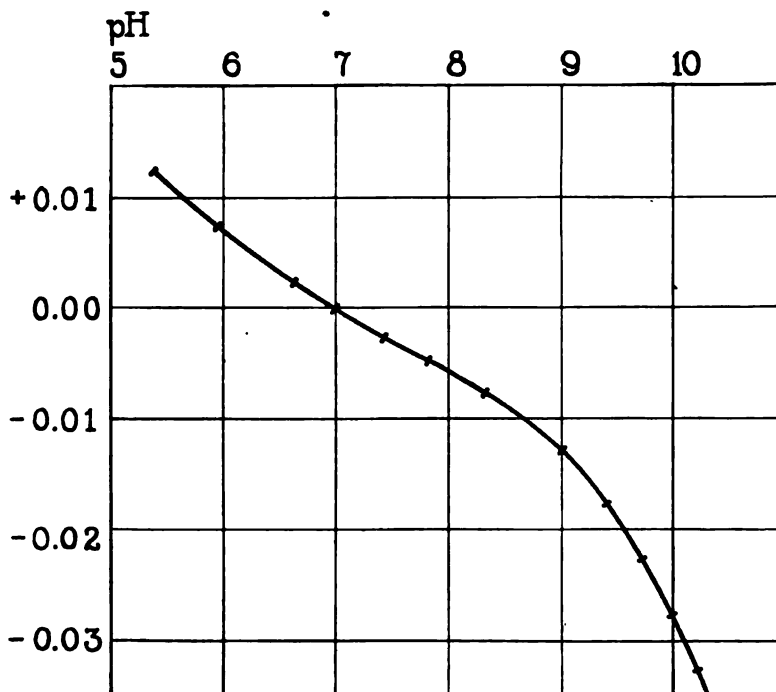


FIG. 3.

Data on the acidity or alkalinity of the ash of foods were taken from Sherman.³ Protein foods leave an acid ash due to oxidation of sulfur and phosphorus and hence meat, eggs, and all foods made of cereals are called "acid-forming." All vegetable foods except cereals contain salts of organic acids and mineral bases that yield alkaline ashes in excess of the acids from the proteins of the same

³ Sherman, H. C., Food products, New York, 1915.

foods. Butter and sugar are practically neutral and milk slightly "base-forming."

The following determinations were made on a man of 75 kilos (L. M.). L. M. was placed on an acid-forming diet for 3 days, then a mixed diet for 3 days, and then a base-forming diet for 3 days. Table I gives the results of the determinations. The other data besides the alkaline reserve are included as they might be thought to indicate something in regard to the food intake and metabolism, but except for the total acidity of the urine they were not planned for this paper. The collection of urine was commenced about 6 hours after the first meal and when blood was drawn on a certain 24 hour period, it was drawn at the end of the period. The alkaline reserve was 0.0335 before beginning the experiment.

TABLE I.

Date.	24 hour urine.			Blood sample.	
	Volume.	Titrateable acidity.	Total N.	Alkaline reserve.	Amino N per 100 cc.
1918	cc.	cc. 0.1 N	gm.	N	mg.
May 8	1,112	436	15.4		4.3 } Acid-forming diet.
" 9	1,200	407	14.9		
" 10	925	417	12.3	0.0335	
" 15	1,125	163	7.3	0.0355	0.7 } Base-forming diet.
" 16	1,360	241	8.8		
" 17	1,375	352	9.7	0.0335	

It may be seen from Table I that the alkaline reserve was not changed by the acid-forming diet from the normal value for this individual (0.0335 N). After 24 hours of base-forming diet the alkaline reserve increased possibly a little more than the limits of error of the method and then went back to normal at the end of 3 days on the base-forming diet. This may have been due to ammonia formation.

Hasselbalch⁴ made some determinations on the CO₂ tension of the alveolar air on acid-forming and base-forming diets and found small differences which if they exceed the limits of error indicate

⁴ Hasselbalch, K. A., *Biochem. Z.*, 1912, xlii, 403.

that the alkaline reserve may be 0.003 N greater on a base-forming than on an acid-forming diet or during fasting. Similar determinations by Blatherwick⁵ show differences of 0.002 N. The method of reduction of CO₂ tensions to alkaline reserve values has already been described.⁶

Van Slyke, Cullen, and Stillman⁷ found that the alkaline reserve of the blood rises during gastric digestion, due to the secretion of HCl from the blood to the stomach. We drew blood at the same stage of gastric digestion (about half hour after luncheon) in each case in the hope of avoiding error due to complication from this source.

In order to use a more easily controlled diet, experiments were made on dogs. Blood was taken from the carotid arteries of four dogs and titrated. The results are given in Table II.

The fifth dog was placed in a cage March 4 and fed on 350 gm. of raw lean beef per day for 2 months and blood drawn from an ear vein at intervals and from the carotid at the end of the experiment. The blood drawn on normal diet and some specimens taken later were discarded due to clotting or getting air in the tube, but samples were correctly drawn on March 13 and May 4 and titrated 0.029 and 0.03 respectively. Since this is the average for the four normal dogs within the errors of the different methods of taking blood, we conclude that it is impossible to lower the bicarbonate concentration or alkaline reserve of dog's blood by a highly acid-forming diet for 2 months.

It was thought that rabbits might be more susceptible to changes in diet since Scott⁸ had shown that the alveolar CO₂ of rabbits varied from 4.57 to 6.3 per cent of an atmosphere. Hasselbalch⁹ gives the pH of rabbit's blood as 7.33 or very nearly what he got for man with his early technique, and, assuming it is the same as man, the above alveolar CO₂ values would place the alkaline

⁵ Blatherwick, N. R., *Arch. Int. Med.*, 1914, xiv, 445.

⁶ McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, xxxi, 519.

⁷ Van Slyke, D. D., Cullen, G. E., and Stillman, E., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 184.

⁸ Scott, F. H., *J. Physiol.*, 1908, xxxvii, 316.

⁹ Hasselbalch, K. A., and Lundsgaard, C., *Skand. Arch. Physiol.*, 1912, xxvii, 31.

reserve at 0.024 to 0.032 *N*. Kuriyama¹⁰ found that food changed the alkaline reserve of rabbit's blood. His figures in our units would be 0.026 *N* on base-forming diet and 0.021 on acid-forming diet. His results are complicated, however, by the fact that he used oats for the latter diet and oats contain no antiscorbutic substance and scurvy has been considered to be associated with acidosis. We wished to avoid this by adding fresh sprouted barley to the oat diet, as sprouted barley cures scurvy in guinea pigs. The rabbits were bled from the carotid artery under ether. The results are shown in Table III.

TABLE II.

Dog No.	Alkaline reserve.
	<i>N</i>
1	0.03
2	0.025
3	0.03
4	0.03

TABLE III.

Rabbit No.	Diet.	Alkaline reserve.
		<i>N</i>
1	Oats and sprouting barley.....	0.0145
2	" " " "	0.0175
3	" " " "	0.007
4	Fasted (water).....	0.0155
5	Carrots and hay.....	0.023
6	" (insufficient number)....	0.013
7	" and cabbage.....	0.021

These rabbits were kept on the diet 1 week except Nos. 1 and 5 which received the diet 9 days and No. 4 which received water but no food for 6 days. The results are not harmonious and the variation may be due to the different amounts of antiscorbutic substances the rabbits received in the period previous to the experiment as well as during the experiment and to partial starvation; *i.e.*, insufficient quantity of food at certain intervals. Thus

¹⁰ Kuriyama, S., *J. Biol. Chem.*, 1918, xxxiii, 215.

No. 4 which fasted showed as low an alkaline reserve as those on acid-forming diet and this is in harmony with the view that fasting induces acidosis or that the rabbit was living on its muscle tissue (as well as fat). It is evident, however, that the alkaline reserve is influenced by diet.

CONCLUSIONS.

The alkaline reserve of man and dog is remarkably resistant to influence of diet, whereas the rabbit is susceptible to the effects of diet and fasting. There is no foundation for the view that the alkaline reserve of man is endangered by acid-forming diets but such diets as usually eaten are deficient in antiscorbutic substances.

NOTE ON THE ULTRAMICROSCOPY OF EGG ALBUMIN.

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The more that is known of the physical chemistry of proteins, the less they appear to resemble the suspension colloids. Sørensen¹ has studied egg albumin from the standpoint of an electrolyte, and considers the molecular weight to be about 3,400, and the pH of the lowest osmotic pressure 4 to 4.4, of the lowest solubility 4.58 (in presence of ammonium sulfate), and of the isoelectric point 4.8. He has shown that it can be purified by recrystallization and that the crystals probably consist of 2 albumin molecules, 3 molecules of sulfuric acid, and 830 water molecules.

If proteins exist in true solution we would expect ultramicroscopic particles to be absent or very numerous, depending on whether the individual molecules could be seen or not. We recrystallized egg albumin three times by Sørensen's method and made a saturated solution of the third crystals in distilled water. The pH of this solution was about 4.2 and the ultramicroscope showed only an occasional submicron. On titrating it to pH=4.8 in a hydrogen electrode by means of 0.1 N NaOH and then re-examining it with the ultramicroscope we found a slight increase in the number of submicrons, but this may be due to the fact (also observed by Sørensen) that a precipitate forms very slowly in such solutions on long standing, and we suppose the submicrons may be the first indication of a change resulting in precipitation or denaturation of the albumin. We believe that in the absence of denatured albumin there would be no submicrons present. The presence of the Tyndall effect can hardly be taken as proving the presence of colloid particles, since it has been observed in solutions of sugars that are not colloids in the usual sense of the term. It seems unfortunate to us that clear solutions of proteins should be classed with suspensoids under the term "colloids."

¹ Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

INDEX TO VOLUME XXXVIII.

- ABDOMINAL** cavity, gas tensions, with some evidence on the diffusion of gases within the body, 71
- Acid**, cinnamic, and derivatives, behavior in the animal body, 7
- , taurocholic, origin, 421
- , uric, endogenous, physiology of, 17
- , —, in urine, colorimetric method for determination, 459
- Acidimetric** titration of grain extracts and amino-acids in the presence of alcohol, 245
- Acidosis**, studies of, 167
- Acids**, bile, bile, taurine, and cholic acid, administration by stomach to show the influence upon bile acid elimination, 379
- , —, quantitative method for analysis, 355
- , —, metabolism of bile, 355, 367, 379, 393, 413, 421
- Age** at which trypsinogen appears in the fetal pancreas, 345
- , heat, and reaction, effect on antiscorbutic foods, 293
- Albumin**, egg, ultramicroscopy of, 549
- Alcohol**, acidimetric titration of grain extracts and amino-acids in the presence of, 245
- Alkaline** reserve of the blood, effect of diet, 539
- ALLEN, FLOYD P.** See **EMMETT** and **ALLEN**, 325
- Amino-acids** and grain extracts in the presence of alcohol, acidimetric titration of, 245
- Ammonia** in blood, determination, 435
- Amylase**, oxidase, peroxidase, and catalase of fresh and dehydrated vegetables, 229
- Analysis**, blood, system of, 81
- ANDO, HIDEZO.** Behavior of cinnamic acid and its derivatives in the animal body, 7
- Antiscorbutic** foods, effect of age, heat, and reaction on, 293
- properties of some milk products, effect of heat on, 305
- BACON**, digestibility of, 43
- Benedict** method, application to the estimation of levulose and inulin, 33
- Bicarbonate** content of plasma, method for titrating, 167
- Bile** acids in dog's bile, quantitative method for analysis, 355
- , —, metabolism of, 355, 367, 379, 393, 413, 421
- , bile acids, taurine, and cholic acid, administration by stomach to show the influence upon bile acid elimination, 379
- fistula dogs, healthy, normal fluctuations, 367
- ingestion and food factors, control, 413
- Biological** analysis of pellagra-producing diets. VI, 113
- observations on the formation of phenol, 13
- test for vitamine, simple, 465
- BIRCKNER, VICTOR.** Acidimetric titration of grain extracts and amino-acids in the presence of alcohol, 245

- BIRCKNER, VICTOR. The zinc content of some food products, 191
- Blood analysis, system of, 81
— and other physiological material, determination of inorganic constituents, 439
—, determination of ammonia, 435
—, effect of diet on alkaline reserve, 539
- BLOUNT, EUGENIA. See FALK, McGUIRE, and BLOUNT, 229
- BLUNT, KATHARINE, and MALLON, MARGUERITE G. Digestibility of bacon, 43
- CATALASE concentration in urine, chyme, and feces, 501
—, oxidase, peroxidase, and amylase of fresh and dehydrated vegetables, 229
- Cholic acid, bile, bile acid, and taurine, administration by stomach to show the influence upon bile acid elimination, 379
- Chyme, urine, and feces, catalase concentration in, 501
- Cinnamic acid and derivatives, behavior in the animal body, 7
- CLARK, E. P. Preparation of rhamnose, 255
- Colormetric determination of the hydrogen ion concentration in small quantities of solution, 49
— method for determination of uric acid in urine, 459
- Copper phosphate method for titration of sugar, 287
- CULLEN, GLENN E. See VAN SLYKE, STILLMAN, and CULLEN, 167
- CULLIGAN, LEO C. See McCLENDON, MYERS, CULLIGAN, and GYDESEN, 535
- DENIS, W., and MINOT, A. S. A method for determination of minute amounts of lead in urine, feces, and tissues, 449
— and —. The non-protein nitrogenous constituents of cow's milk, 453
- Determination, colorimetric, of hydrogen ion concentration in small quantities of solution, 49
— of ammonia in blood, 435
— — levulose and inulin, application of Benedict method to, 33
— — minute amounts of lead in urine, feces, and tissues, method for, 449
— — the inorganic constituents of blood and other physiological material, 439
— — urea in urine by direct Nesslerization, 111
— — urea in urine, rapid method, 57
— — uric acid in urine by colorimetric method, 459
- Diet, effect on alkaline reserve of the blood, 539
- Diets, pellagra-producing, biological analysis, 113
- Diffusion of gases within the body, gas tensions of the abdominal cavity, with some evidence on, 71
- Digestibility of bacon, 43
- EGG albumin, ultramicroscopy of, 549
- Elimination, bile acid, administration by stomach of bile, bile acids, taurine, and cholic acids to show the influence upon, 379
- EMMETT, A. D., and ALLEN, FLOYD P. Nutritional studies on the growth of frog larvæ (*Rana pipiens*). First paper, 325

- EMMETT, A. D., and LUKOS, G. O.
Is lactalbumin a complete protein for growth, 147
— and —. The absence of fat-soluble A vitamins in certain ductless glands, 441
— and —. — stability of lactalbumin toward heat, 257
Endogenous and exogenous factors of metabolism of bile acids, 393
— uric acid, physiology of, 17
ENGSTRAND, O. J. See McCLENDON, VON MEYSENBUG, ENGSTRAND, and KING, 539
Enzyme actions, studies, 229
Enzymes, pancreatic, relationship of, 487
Exogenous and endogenous factors of metabolism of bile acids, 393
- FALK, K. GEORGE, MCGUIRE, GRACE, and BLOUNT, EUGENIA.
Studies on enzyme action. XVII. The oxidase, peroxidase, catalase, and amylase of fresh and dehydrated vegetables, 229
Fat-soluble A vitamins, absence in certain ductless glands, 441
Feces, urine, and chyme, catalase concentration in, 501
—, —, — tissues, method for determination of minute amounts of lead in, 449
FENGER, FREDERIC, and HULL, MARY. Relationship of the pancreatic enzymes, 487
Fetal pancreas, age at which trypsinogen appears, 345
FINKS, A. J. See JOHNS and FINKS, 63
Fistula dogs, normal bile, normal fluctuations, 367
Foam breaker, use of turpentine resin in turpentine, 529
FOLIN, OTTO, and PECK, EUGENE C. A revision of the copper phosphate method for the titration of sugar, 287
FOLIN, OTTO, and WRIGHT, L. E.
A simplified macro-Kjeldahl method for urine, 461
— and WU, HSIEN. A revised colorimetric method for determination of uric acid in urine, 459
— and —. A system of blood analysis, 81
— and YOUNGBERG, GUY E. Note on the determination of urea in urine by direct Nesslerization, 111
Food factors and bile ingestion, control, 413
Foods, hydrogen ion concentration of, 531
FOSTER, M. G., and HOOPER, C. W.
The metabolism of bile acids. I. A quantitative method for analysis of bile acids in dog's bile, 355
—, HOOPER, C. W., and WHIPPLE, G. H. The metabolism of bile acids. II. Normal fluctuations in healthy bile fistula dogs, 367. III. Administration by stomach of bile, bile acids, taurine, and cholic acid to show the influence upon bile acid elimination, 379. IV. Endogenous and exogenous factors, 393. V. Control of bile ingestion and food factors, 413. VI. Origin of taurocholic acid, 421
Furan and hydrofuran derivatives in the animal organism, metabolism of, 1
- GAS tensions in the tissues of the mouth, 67
— — of the abdominal cavity, with some evidence on the diffusion of gases within the body, 71

- Gelatin, effect of hydrogen ion concentration on liquefaction of, 179
- Glands, certain ductless, absence of fat-soluble A vitamine, 441
- GREENWALD, ISIDOR. A note on the determination of the inorganic constituents of blood and other physiological material, 439
- Growth of frog larvæ (*Rana pipiens*), nutritional studies, 325
- GYDESEN, CARL S. See McCLENDON, MYERS, CULLIGAN, and GYDESEN, 535
- H**AAS, A. R. C. Colorimetric determination of the hydrogen ion concentration in small quantities of solution, 49
- HAGGARD, HOWARD W., and HENDERSON, YANDELL. Gas tensions of the abdominal cavity, with some evidence on the diffusion of gases within the body, 71
- HART, E. B., and HUMPHREY, G. C. Can "home grown rations" supply proteins of adequate quality and quantity for high milk production, 515
- and STEENBOCK, H. Maintenance and production value of some protein mixtures, 267
- , STEENBOCK, H., and SMITH, D. W. Studies of experimental scurvy. Effect of heat on the antiscorbutic properties of some milk products, 305
- Heat, age, and reaction, effect on antiscorbutic foods, 293
- , effect on antiscorbutic properties of some milk products, 305
- , stability of lactalbumin toward, 257
- HENDERSON, YANDELL, and STEHLE, RAYMOND L. Gas tensions in the tissues of the mouth, 67
- See HAGGARD and HENDERSON, 71
- HESS, ALFRED, F., and UNGER, LESTER J. The scurvy of guinea pigs. III. The effect of age, heat, and reaction on antiscorbutic foods, 293
- HILTNER, R. S., and WICHMAN, H. J. Zinc in oysters, 205
- HOOPER, C. W. See FOSTER and HOOPER, 355
- See FOSTER, HOOPER, and WHIPPLE, 367, 379, 393, 413, 421
- Hordein, lysine as a hydrolytic product of, 63
- HÖST, H. F. A study of the physiology of endogenous uric acid, 17
- HULL, MARY. See FENGER and HULL, 487
- HUMPHREY, G. C. See HART and HUMPHREY, 515
- Hydrofuran and furan derivatives in the animal organisms, metabolism of, 1
- Hydrogen ion concentration, colorimetric determination in small quantities of solution, 49
- — —, effect on liquefaction of gelatin, 179
- — — of foods, 531
- — — the ileum, factors influencing, 535
- Hydrolytic product of hordein, lysine, 63
- I**LEUM, factors influencing the hydrogen ion concentration of, 535
- Inorganic constituents of blood and other physiological material, determination, 439

- Inulin and levulose, application of Benedict method to the estimation of, 33
—, behavior in the animal body, 33
- JAHR, H. M.** See **MORGULIS** and **JAHR**, 435
- JOHNS, CARL O.**, and **FINKS, A. J.**
Lysine as a hydrolytic product of hordein, 63
- JOHNSON, ALFRED J.** See **PATTEN** and **JOHNSON**, 179
- KENDALL, E. C.** The use of turpentine resin in turpentine as a foam breaker, 529
- KING, FRANCES.** See **McCLENDON**, **VON MEYSENBUG**, **ENGSTRAND**, and **KING**, 539
- Kjeldahl, macro-**, method for urine, 461
- LACTALBUMIN** as a complete protein for growth, 147
—, stability toward heat, 257
- Lead** in urine, feces, and tissues, method for determination of minute amounts, 449
- Levulose** and **inulin**, application of Benedict method to the estimation of, 33
- LUROS, G. O.** See **EMMETT** and **LUROS**, 147, 257, 441
- Lysine** as a hydrolytic product of hordein, 63
- MAINTENANCE** and production value of some protein mixtures, 267
- MALLON, MARGUERITE G.** See **BLUNT** and **MALLON**, 43
- McCLENDON, J. F.**, and **PRENDERGAST, H. J.** Note on the ultramicroscopy of egg albumin, 549
—, and **SHARP, PAUL F.** The hydrogen ion concentration of foods, 531
- McCLENDON, J. F.**, **MYERS, FRANK J.**, **CULLIGAN, LEO C.**, and **GYDESEN, CARL S.** Factors influencing the hydrogen ion concentration of the ileum, 355
—, **VON MEYSENBUG, L.**, **ENGSTRAND, O. J.**, and **KING, FRANCES.** Effect of diet on the alkaline reserve of the blood, 539
- McCOLLUM, E. V.**, **SIMMONDS, N.**, and **PARSONS, H. T.** Biological analysis of pellagra-producing diets. VI. Observations on the faults of certain diets comparable to those employed by man in pellagrous districts, 113
- McGUIRE, GRACE.** See **FALK, McGUIRE**, and **BLOUNT**, 229
- MENDEL, LAFAYETTE B.** See **OSBORNE** and **MENDEL**, 223
- Metabolism** of bile acids, 355, 367, 379, 393, 413, 421
— — the furan and hydrofuran derivatives in the animal organism, 1
- Method, Benedict**, application to the estimation of levulose and inulin, 33
—, colorimetric, for determination of uric acid in urine, 459
— for determination of minute amounts of lead in urine, feces, and tissues, 449
— — titrating the bicarbonate content of the plasma, 167
—, macro-Kjeldahl, for urine, 461
—, quantitative, for analysis of bile acids in dog's bile, 355
—, rapid, for estimation of urea in urine, 57
—, revision of copper phosphate, for the titration of sugar, 287
- VON MEYSENBUG, L.** See **McCLENDON**, **VON MEYSENBUG**, **ENGSTRAND**, and **KING**, 539

- Milk, cow's, non-protein nitrogenous constituents, 453
 —, high production, can "home grown rations" supply proteins of adequate quality and quantity, 515
 — products, effect of heat on antiscorbutic properties of, 305
- MINOT, A. S. See DENIS and MINOT, 449, 453
- MORGULIS, SERGIUS, and JAHR, H. M. Determination of ammonia in the blood, 435
- Mouth, gas tensions in tissues of, 67
- MYERS, FRANK J. See McCLENDON, MYERS, CULLIGAN, and GYDESEN, 535
- NESSLERIZATION, direct, determination of urea in urine, 111
- Nitrogenous, non-protein constituents of cow's milk, 453
- NORGAARD, A. V. S. Studies of the concentration of catalase in urine, chyme, and feces, 501
- Nutritional studies on the growth of frog larvæ (*Rana pipiens*), 325
- Nutritive value of yeast protein, 223
- OKEY, RUTH. Studies on the behavior of inulin in the animal body. Preliminary paper. Application of the Benedict method to the estimation of levulose and inulin, 33
- OSBORNE, THOMAS B., and MENDEL, LAFAYETTE B. The nutritive value of yeast protein, 223
- Oxidase, peroxidase, catalase, and amylase of fresh and dehydrated vegetables, 229
- PANCREAS, fetal, age at which trypsinogen appears, 345
- Pancreatic enzymes, relationship of, 487
- PARSONS, H. T. See MCCOLLUM, SIMMONDS, and PARSONS, 113
- PATTEN, HARRISON E., and JOHNSON, ALFRED J. The effect of hydrogen ion concentration on the liquefaction of gelatin, 179
- PECK, EUGENE C. See FOLIN and PECK, 287
- Pellagra-producing diets, biological analysis, 113
- Pellagrous districts, observations on the faults of certain diets comparable to those employed by man in, 113
- Peroxidase, oxidase, catalase, and amylase of fresh and dehydrated vegetables, 229
- Phenol, biological observations on formation of, 13
- Phosphate, copper, method for titration of sugar, 287
- Physiological material, other, and blood, determination of inorganic constituents, 439
- Physiology of endogenous uric acid, 17
- Plasma, method for titrating bicarbonate content of, 167
- PRENDERGAST, H. J. See McCLENDON and PRENDERGAST, 549
- Production and maintenance values of some protein mixtures, 267
- Protein complete for growth, lactalbumin, 147
 — mixtures, maintenance and production value of, 267
 —, non-, nitrogenous constituents of cow's milk, 453
 —, yeast, nutritive value, 223
- Proteins in "home grown rations" of adequate quality and quantity for high milk production, 515

- Proteins, serum, in albino rats at different ages, 161
- REACTION**, age, and heat, effect on antiscorbutic foods, 293
- Reserve of the blood, alkaline effect of diet, 539
- Resin, turpentine, in turpentine as a foam breaker, 529
- Rhamnose, preparation of, 255
- SAMPSON**, JOHN J. The age at which trypsinogen appears in the fetal pancreas, 345
- Scurvy, experimental, 305
- of guinea pigs, 293
- Serum proteins in albino rats at different ages, 161
- SHARP**, PAUL F. See **McCLENDON** and **SHARP**, 531
- SIMMONDS**, N. See **McCOLLUM**, **SIMMONDS**, and **PARSONS**, 113
- SMITH**, D. W. See **HART**, **STEENBOCK**, and **SMITH**, 305
- Solution, small quantities, colorimetric determination of hydrogen ion concentration, 49
- STEENBOCK**, H. See **HART** and **STEENBOCK**, 267
- See **HART**, **STEENBOCK**, and **SMITH**, 305
- STEHLE**, RAYMOND L. See **HENDERSON** and **STEHLE**, 67
- STILLMAN**, EDGAR. See **VAN SLYKE**, **STILLMAN**, and **CULLEN**, 167
- Sugar, a revision of the copper phosphate method for the titration of, 287
- SUMNER**, JAMES B. A rapid method for the estimation of urea in urine, 57
- SUZUKI**, NOBUYOSHI. Metabolism of the furan and hydrofuran derivatives in the animal organism, 1
- System of blood analysis, 81
- TAURINE**, bile, bile acids, and cholic acid, administration by stomach to show the influence upon bile acid elimination, 379
- Taurocholic acid, origin, 421
- Test for vitamine, simple biological, 465
- Tissues, urine, and feces, method for determination of minute amounts of lead in, 449
- Titration, acidimetric, of grain extracts and amino-acids in the presence of alcohol, 245
- of bicarbonate content of plasma, method, 167
- — — sugar, a revision of the copper phosphate method, 287
- TOYAMA**, IKUZO. Relative abundance of serum proteins in albino rats at different ages, 161
- Trypsinogen in fetal pancreas, 345
- TSUDJI**, MIDORI. Biological observations on the formation of phenol, 13
- Turpentine resin in turpentine as a foam breaker, 529
- ULTRAMICROSCOPY** of egg albumin, 549
- UNGER**, LESTER J. See **Hess** and **UNGER**, 293
- Urea in urine, determination by direct Nesslerization, 111
- — —, rapid method for estimation, 57
- Uric acid, endogenous, physiology of, 17
- — — in urine, colorimetric method for determination, 459
- Urine, chyme, and feces, catalase concentration in, 501
- , colorimetric method for determination of uric acid in, 459
- , feces, and tissues, method for determination of minute amounts of lead in, 449

- Urine, macro-Kjeldahl method for, 461
—, rapid method for estimation of urea in, 57
—, urea in, determination by direct Nesslerization, 111
- VAN SLYKE, DONALD D., STILLMAN, EDGAR, and CULLEN, GLENN E.** Studies of acidosis. XIII. A method for titrating the bicarbonate content of the plasma, 167
- Vegetables, fresh and dehydrated, oxydase, peroxydase, catalase, and amylase of, 229
- Vitamine, fat-soluble A, absence in certain ductless glands, 441
— requirement of yeast, 465
—, simple biological test for, 465
- WHIPPLE, G. H.** See **FOSTER, HOOPER, and WHIPPLE**, 367, 379, 393, 413, 421
- WICHMANN, H. J.** See **HILTNER and WICHMANN**, 205
- WILLIAMS, ROGER J.** The vitamine requirement of yeast. A simple biological test for vitamine, 465
- WRIGHT, L. E.** See **FOLIN and WRIGHT**, 461
- WU, HSIEN.** See **FOLIN and WU**, 81, 459
- YEAST** protein, nutritive value, 223
—, vitamine requirement of, 465
- YOUNGBERG, GUY E.** See **FOLIN and YOUNGBERG**, 111
- ZINC** content of some food products, 191
— in oysters, 205

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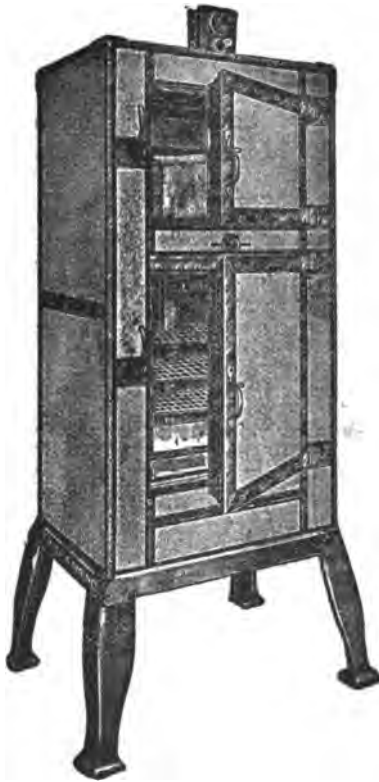
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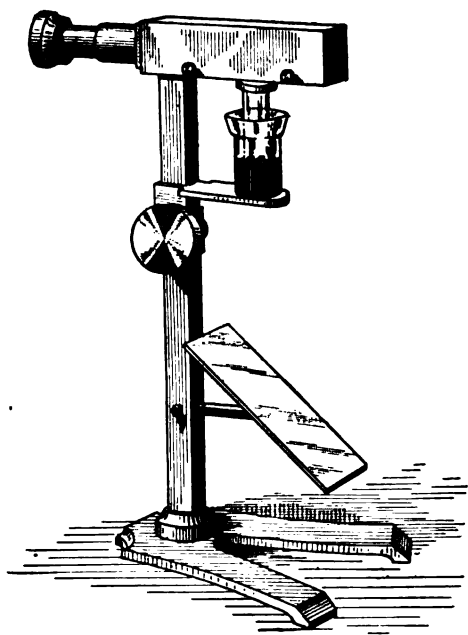
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CONTENTS OF VOL. I, No. 5, MAY 20, 1919

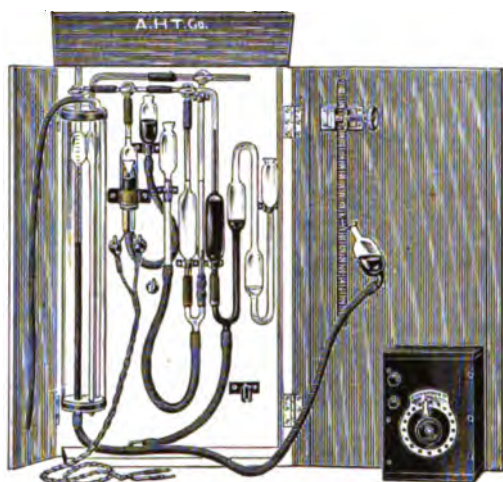
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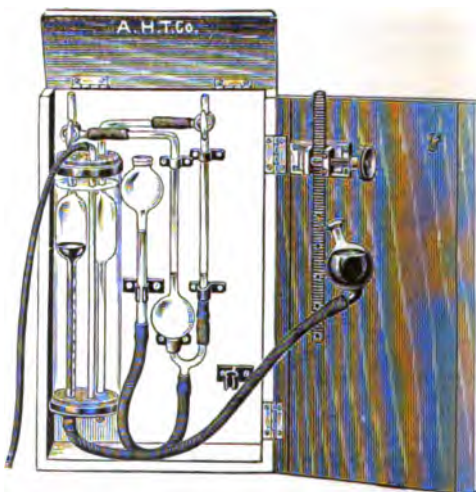
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CONTENTS

MORGULIS, SERGIUS, and JAHR, H. M. Determination of ammonia in the blood	41
GREENWALD, ISIDOR. A note on the determination of the inorganic constituents of blood and other physiological material.....	41
EMMETT, A. D., and LUKOS, G. O. The absence of fat-soluble A vitamins in certain ductless glands.....	41
DENIS, W., and MINOT, A. S. A method for determination of minute amounts of lead in urine, feces, and tissues.....	41
DENIS, W., and MINOT, A. S. The non-protein nitrogenous constituents of cow's milk ..	41
FOLIN, OTTO, and WU, HSIEN. A revised colorimetric method for determination of uric acid in urine.....	41
FOLIN, OTTO, and WRIGHT, L.E. A simplified macro-Kjeldahl method for urine.....	41
WILLIAMS, ROGER J. The vitamin requirement of yeast. A simple biological test for vitamin. Plate 6.....	41
FENGER, FREDERIC, and HULL, MARY. Relationship of the pancreatic enzymes.....	48
NORGAARD, A. V. S. Studies of the concentration of catalase in urine, chyme, and feces....	51
HART, E. B., and HUMPHREY, G. C. Can "home grown rations" supply proteins of adequate quality and quantity for high milk production.....	51
KENDALL, E. C. The use of turpentine resin in turpentine as a foam breaker.....	51
McCLENDON, J. F., and SHARP, PAUL F. The hydrogen ion concentration of foods.....	51
McCLENDON, J. F., MYERS, FRANK J., CULLIGAN, LEO C., and GYDESEN, CARL S. Factors influencing the hydrogen ion concentration of the ileum.....	51
McCLENDON, J. F., VON MEYSENBUG, L., ENGSTRAND, O. J., and KING, FRANCES. Effect of diet on the alkaline reserve of the blood.....	51
McCLENDON, J. F., and PRENDERGAST, H. J. Note on the ultramicroscopy of egg albumin.....	51
Index to Volume XXXVIII.....	51

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